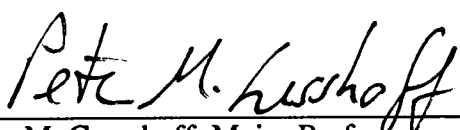
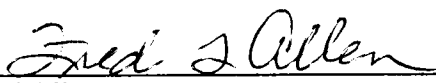


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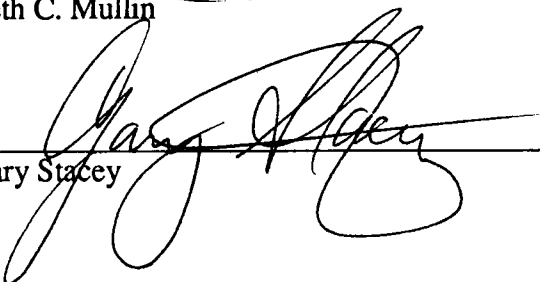
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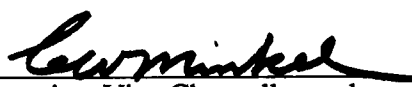
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***AGROBACTERIUM RHIZOGENES*-MEDIATED
PLANT ROOT TRANSFORMATION FOR STUDY OF
GENES IMPORTANT IN LEGUME NODULATION**

A Dissertation
Presented for the
Doctor of Philosophy
Degree
The University of Tennessee, Knoxville

Ru-Ju Chian

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ABSTRACT

Nodulation is the consequence of the specific interaction between legume plants and the symbiotic bacteria *Rhizobium* (or its relatives *Bradyrhizobium* and *Azorhizobium*). Nodules provide a specific environment for bacterial growth and nitrogen-fixation. Although induced by bacteria or bacterial-derived compounds, (i.e., Nod factors) organogenesis of nodules is directed by plant genes. Many cellular activities during nodule development are fundamental to plant growth, such as dedifferentiation of mature cells, induction of cell division, regulation of plant hormones, shoot-root communications, etc. Thus the study of nodulation is not only important to agricultural practice, but also provides an ideal system to address questions concerning plant-microbe interactions and plant developmental biology.

To investigate gene regulation in nodule development using molecular biological tools, we established *Agrobacterium rhizogenes*-mediated transformation systems for soybean and *Lotus japonicus*, and regeneration protocols for *L. japonicus*. Plant genes important in nodule initiation and cell division were isolated. The ultimate goal of this project is to develop a system to study regulation of these genes in plants utilizing plant transformation. Part I of the thesis introduces general aspects of nodulation and *Agrobacterium*-mediated transformation. High frequency soybean root transformation based on *A. rhizogenes* strain K599 is reported in Part II. Meristematic nodules were harvested from hairy roots of different soybean cultivars and their N₂-fixing activity was re-examined. The possible role of agrobacterial genes integrated into plant genome in interfering with programmed organ development is discussed. Part III introduces *A. rhizogenes*-mediated root transformation of model legume *Lotus japonicus*. This work reflects a logic transition of transformation from soybean to *Lotus* accompanied with advances in nodulation research. High frequency root transformation and simple and fast regeneration of transgenic *L. japonicus* plants were demonstrated. A brief comparison between the two systems was given. Isolation of a special early nodulin gene, the genomic clone of *enod40* gene from *L. japonicus* is presented in Part IV. This gene is particularly interesting because its potential role in regulation of nodule induction and intracellular hormone balance. A division of all *enod40* clones into two groups is proposed, based on sequence comparison. Because of the advanced *L. japonicus* root transformation and regeneration system, it has also become feasible to study gene functions in a homologous

system with a model legume. Throughout the course of this research, the involvement of plant hormones, with relation to bacterial oncogenes (such as *rol* genes of *A. rhizogenes*) and plant regulatory genes (such as *enod40*), in morphogenesis of different organs were frequently surfaced. Part V integrates our knowledge from diverse research areas into relatively simple models which can help interpret some results observed in this study and stimulate thoughts for future studies.

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ABBREVIATIONS

bp	base pair
CaMV	Cauliflower Mosaic Virus
cDNA	complementary DNA
cfu	colony forming unit
cm ²	square centimeter
cpm	counts per minute
dATP	deoxy adenosine tri-phosphate
DNA	deoxy ribonucleic acid
dNTP	deoxy nucleotide tri-phosphate
EDTA	[Ethylenedinitrilo]-Tetraacetic acid
EST	Expressed sequence tag
g _n	Centrifugation force
kb	kilo base-pairs
M	Molar (concentration)
MS	Murashige-Skoog medium
NCBI	National Center for Biotechnology Information
NPA	N-1-(naphthyl)phthalamic acid (auxin transport inhibitor)
<i>nts</i>	nitrate tolerant symbiosis (synonomous to supernodulation)
ORF	Open reading frame
PCR	Polymerase chain reaction
pfu	plaque forming unit
<i>rol</i>	root locus (<i>Agrobacterium rhizogenes</i> oncogene)
rpm	revolutions per minute
SDS	sodium dodecyl sulfate
SM	suspension medium
T-DNA	transferred DNA (of <i>Agrobacterium</i>)
TIBA	2,3,5-triiodobenzoic acid (auxin transport inhibitor)
wt	wild type
YEM	Yeast extract-mannitol medium

Part I

**INTRODUCTION AND LITERATURE REVIEW:
LEGUME NODULATION AND *AGROBACTERIUM*-MEDIATED PLANT
TRANSFORMATION**

Nitrogenous compounds utilized by plants come from three major sources: synthetic and natural fertilizers, nitrite and nitrate brought to soil by lightning and symbiotic nitrogen fixation. Conversion of nitrogen gas (N_2) to the reduced, plant-usable forms via synthetic fertilizers is a high energy-consuming process, therefore, economic alternative solutions are desirable. This is especially true in developing countries where the cost of synthetic fertilizers limits their widespread use, and farmers are largely dependent on natural resources.

Symbiotic nitrogen fixation is a naturally occurring nitrogen conversion process, resulting from the interactions between a special group of bacteria and legume plants. Although some free-living bacteria can fix atmospheric nitrogen, they do this only in limited amounts and do not contribute significantly to plant growth. Considerable quantities of nitrogen are converted by species of the bacterial genera *Rhizobium*, *Bradyrhizobium* and *Azorhizobium* in plant organs called nodules. The mutual relationship is beneficial to both partners in the interaction. Legume plants provide bacteria with a special environment and supply them with carbohydrates. Bacteria (in their transformed form, bacteroids) "pay" plants back with ammonia, the reduced form of nitrogen. Because the nodule is essential for bacteroid function, nodulation studies constitute an essential part of symbiotic nitrogen fixation research.

Legume nodulation

It is known now that many bacteria are capable of fixing N_2 (e.g., actinomycetes, *Anabaena*, some species of *Pseudomonas*, etc). Of these, *Rhizobium* and *Bradyrhizobium* are most common in agricultural niches and convert atmospheric N_2 into ammonia at an agronomically significant scale. As such, the genetics, physiology and biochemistry of bacteria in the two genera and their interactions with legume plants have been studied extensively. Throughout this dissertation, unless otherwise mentioned, *Rhizobium* is used to encompass all agriculturally important N-fixing species belonging to *Rhizobium*, *Bradyrhizobium*, *Mesorhizobium*, *Azorhizobium* and *Sinorhizobium* groups.

FORMATION OF ROOT NODULES

Nodules on legumes are induced by *Rhizobium*. It is now clear that to elicit nodules on plant roots, a package of (*Brady*)*rhizobium* genes has to be expressed (Franssen *et al.*, 1992; Long, 1996). The common theme in the establishment of a symbiotic relationship (Figure 1.1) is the induction of the *Rhizobium* nodulation regulatory genes, the *nodD*s, by flavonoids or isoflavonoids in exudates of legume roots (Redmond *et al.*, 1986; Peters *et al.*, 1986). The *nodD* gene products in turn activate other *nod* genes to give rise to the biosynthesis of specific lipo-chitin-oligosaccharides (LCOs) also known as Nod factors

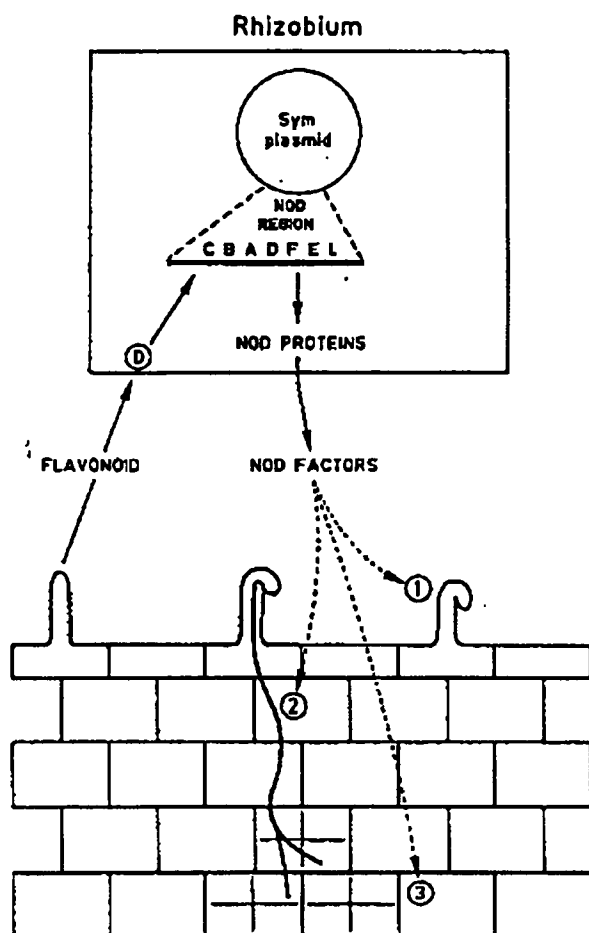


Figure 1.1. A schematic diagram of *Rhizobium*-legume interaction. Plant secreted (iso)flavonoids activate bacterial *nod* genes, resulting in biosynthesis of the Nod factors. Nod factors induce multiple cellular responses, including (1) root hair curling and deformation, (2) *Rhizobium* invasion-related events and (3) cortical cell divisions. (Adapted from Franssen *et al.*, 1992).

(Lerouge *et al.*, 1990; Clarke *et al.*, 1992). At picomolar to subfemtomolar concentration (10^{-10} to $< 10^{-12}$ M) Nod factors trigger multiple cellular responses to nodule induction and rhizobia invasion (Long, 1996; Carlson *et al.*, 1993) in spatially separated locations. The observed changes at tissue, cell and subcellular levels include membrane depolarization (Ehrhardt *et al.*, 1992), root hair curling and deformation, cell division (John *et al.*, 1997) and preconditioning cells for infection thread penetration in the cortex (Yang *et al.*, 1994). Cortical cell division is accompanied by divisions in pericycle cells, which provide nodule primordia with a peripheral vascular system. Activation of a set of early nodulin genes by Nod factors was detected (van Rhijn *et al.*, 1997; Minami *et al.*, 1996) in root pericycle, root cortex and nodule primordia (Geurts and Franssen, 1996; Kijne, 1992; Yang *et al.*, 1994;

Minami *et al.*, 1996). All these changes apparently prepare roots for nodule formation and bacterial invasion. Bacteria invade root hair cells by dissolving the host cell wall and stimulating host cell plasma membrane invagination. As invasion proceeds, the plant-originated plasma membrane components continue to be deposited on the tip of infection threads which grow into the cortex. When infection threads, guided by a preinfection bridge, have penetrated into nodule primordia, bacteria are released and surrounded by host cell synthesized symbiosome membrane, and later transformed into N-fixing bacteroids.

The fact that nodules can be induced by treating roots with Nod factors alone indicates that nodule morphogenesis is controlled by plant genes only. However, formation of functional nodules depends on constant communications between rhizobia and host plant (Geurts and Franssen, 1996). Infection thread formation and symbiotic functions require the presence of bacteria, as well as exchange of information and metabolites between the two partners. Bacterial mutants which affect infection thread formation, rhizobial release from infection threads and differentiation of nodules have been assigned to particular genes (Barbour *et al.*, 1992; Djordevic *et al.*, 1987; Dylan *et al.*, 1990). Similarly, plant mutants which block rhizobia invasion, and nodule formation at different stages have been identified (Caetano-Anollés and Gresshoff, 1991; Utrup *et al.*, 1993).

In plant development, morphogenesis of leaves, flowers, roots and other organs has been long recognized as preprogrammed processes (Meyerowitz, 1997). The fate of organs and tissues are decided in the early stage of embryogenesis (van den Berg *et al.*, 1995; Sheres *et al.*, 1996). Although mature tissues and cells can dedifferentiate and regenerate into a whole plant, totipotency occurs mostly when artificial conditions are superimposed (Reinert and Backs, 1968; Vasil and Vasil, 1972; Schell *et al.*, 1993). Unlike normal organogenesis, nodule initiation is from differentiated cells. This process is dictated by a specific signal exchange between bacteria and host cells. Plant genes control subsequent progression of nodule growth, but ambient environmental conditions, such as heat, salinity, nitrate and acidity, can have a strong influence on this process. For example, high nitrate concentration inhibits nodulation (Carroll and Gresshoff, 1983). In addition, an internal regulatory mechanism, that controls the number/mass of nodules, apparently exists (Nutman, 1952; Miao and Verma, 1993; Nodari *et al.*, 1993). Shoot-root communication has been clearly demonstrated to be part of such a mechanism (Gresshoff, 1993; Caetano-Anollés and Gresshoff, 1990; Delves *et al.*, 1986). Nodule-root dialogue can be expected

as well. Because of the inducibility and plasticity, symbiotic nodulation in legume plants provides an unparalleled system for developmental biology and plant-microbe interactions, open to genetic, biochemical and physiological studies.

During the initiation of nodule development, cell divisions occur in the inner (in temperate legumes which usually produce indeterminate nodule) or sub-epidermal (in tropical legumes which in general give rise to determinate nodules) cortical cells opposite to the protoxylem pole. These cell division loci develop into nodule primordia. Several early nodulin genes, e.g., *enod40* (Kouchi and Hata, 1993; Yang *et al.*, 1993), *enod12* (Sheres *et al.*, 1990; Pichon *et al.*, 1992), *Rhizobium*-induced peroxidase (*rip*, Cook *et al.*, 1995) and genes involved in cell division cycle such as *cdc2* (Miao *et al.*, 1993), cyclins (Hata *et al.*, 1991; Kouchi *et al.*, 1995) are expressed at this stage. Concomitant with the expression of nodulation-associated and cell cycle-specific genes, some metabolic pathways and genes involved in divergent cellular activities are activated (Zhang and Chollet, 1997; Takane *et al.*, 1997). Among them there are phenylalanine ammonia lyase (PAL) and chalcone synthase (CHS) in the flavonoid biosynthesis pathway (Estabrook and Sengupta-Gopalan, 1991; Recourt *et al.*, 1992; Perlick *et al.*, 1996), and ethylene biosynthesis genes (Heidstra *et al.*, 1997).

NODULINS AND THEIR BIOLOGICAL FUNCTIONS

Nodulins were originally defined as plant proteins found specifically in nodules (Nap and Bisseling, 1990). According to the timing of expression, they are divided into early and late nodulins. Early nodulins are expressed in nodule primordia preceding the onset of nitrogen fixation while late nodulins are expressed after the onset of nitrogen fixation. Most late nodulin genes discovered to date have been assigned specific functions. For example, *lbc3* encodes a leghemoglobin involved in transport and sequestration of oxygen molecules which are toxic to nitrogenase in the symbiosome. The product of *nod26* forms an ion channel protein (Rivers *et al.*, 1997; Weaver *et al.*, 1994) while that of *enod24* is a peribacteroid membrane protein (Cheon *et al.*, 1994). Uricase which is responsible for synthesis of transportable form of reduced nitrogen, allantoin and allantoic acid, is expressed by *nod35* (Takane *et al.*, 1997). A nodule-specific sucrose synthase is encoded by nodulin-100 (Thummler and Verma, 1987; Zhang and Chollet, 1997).

In contrast, no clear role has been illustrated for any of the early nodulins. Based on the temporal and spatial patterns of gene expression, as well as the deduced structure of gene products, the functions of some early nodulins have been suggested. Furthermore, it has been found that some of the early nodulin genes also exist in other tissues and organs, and in non-legume plants. ENOD12, a proline-rich protein may be a cell wall component and is suspected of playing a role in the regulation of oxygen content in infected cells. However, this gene was lately found also to be expressed in stems and flowers (Sheres *et al.*, 1990; Hirsch and LaRue, 1997). Some *Medicago* subspecies lacking this gene nodulate and flower normally (Csanadi *et al.*, 1994). Similarly, *enod40*, which responds to rhizobia infection or Nod factor treatment in root pericycle cells, cortical dividing cells (Charon *et al.*, 1997) and nodule primordia, was also identified from the non-legume tobacco (van de Sande *et al.*, 1996). Thus some nodulins may not be essential to nodulation, but may be involved in general cellular responses to stimuli. For instance, a *Lotus japonicus* nodulin was identified as a protein phosphatase 2C (PP2C, K. Szczygowski, personal communication), a member of an enzyme family whose members are involved in a broad range of cellular activities. Such dilemma was also found with leghemoglobin, which regulates oxygen content in infected nodule cells for nitrogenase functioning, as it appears to be ubiquitous in the plant kingdom (Jacobsen-Lyon *et al.*, 1995; Doyle and Doyle, 1997; Bogusz *et al.*, 1988). As yet, it has been impossible to find a mutation in a nodulin gene that results in a mutant phenotype. In parallel, none of the plant mutations known to affect morphology, development or function has been explained by an alteration of a nodulin.

***Agrobacterium*-mediated plant transformation**

Nodule organogenesis and development is an extremely complicated process. The involvement and requirement for plant-bacterial communication and signal exchange are now well documented. Plant hormones, auxin, cytokinin and ethylene are apparently involved (Hirsch and Fang, 1994). Full comprehension of the process, however, requires a thorough understanding of the function of genes or gene products from both partners, and their interactions. This goal can be pursued through molecular approaches. Gene isolation, mutation (by base pair substitutions, deletions) and silencing (by antisense constructs) are commonly used in plant studies (Flavell, 1994; Hawkins and Nellen, 1994), but all these maneuvers cannot provide meaningful information without transformation techniques.

To reveal its function, a manipulated gene needs to be reintroduced into plant cells and its effects analyzed through alterations of phenotype in transformed plants or cells. To approach this, systems to deliver foreign genes are necessary. Several methods have been exploited for the purpose, e.g., biolistic or particle bombardment, electroporation or chemical treatment of protoplasts, and *Agrobacterium*-mediated transformation (agroinfection). The biolistic methods deliver foreign genes utilizing high speed (accelerated in an electric field or pressurized wave) microparticles, such as gold or tungsten particles, coated with naked DNA to be transferred. Both electroporation and chemical methods are aimed to increase the plasma membrane permeability of host cells by electric current or polyethylene glycol (PEG)/calcium phosphate, thereby facilitating DNA uptake (Lazzeri, 1995; Ye *et al.*, 1990).

Agrobacterium species are natural plant pathogens of many dicotyledonous plants.

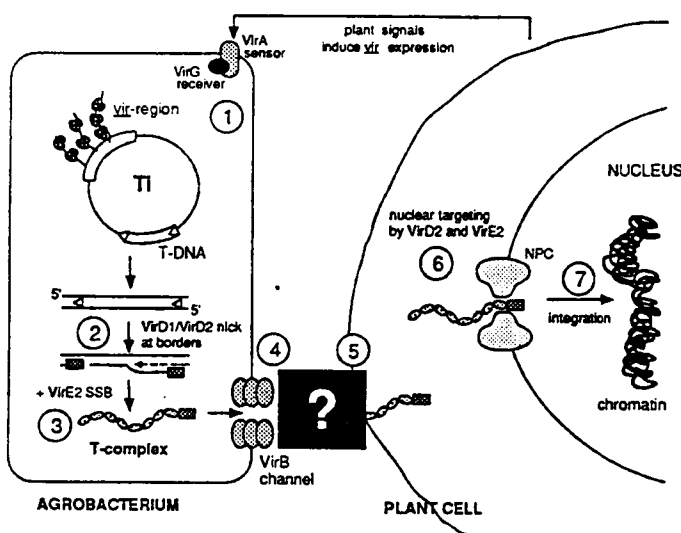


Figure 1.2. *Agrobacterium*-mediated plant transformation. T-DNA of Ti (*A. tumefaciens*) or Ri (*A. rhizogenes*) plasmid defined by the border sequence (open arrow-heads) is modified and used as a vehicle to deliver genes of interest into plant cells. Many Vir proteins are involved in the synthesis and transfer of the single stranded T-DNA. The VirD2 and VirE2 maintain the T-DNA in transportable form. They also contain nuclear targeting signal for transporting T-DNA into plant nuclei. The involvement of Vir protein in T-DNA integration is not clear yet. (Adapted from McLean *et al.*, 1993).

Two species are predominantly used in plant genetic engineering. *A. tumefaciens* causes tumor (crown gall) symptoms while *A. rhizogenes* induces abundant adventitious roots (hairy roots, Cararelli *et al.*, 1987). Both abnormalities are caused by transfer of bacterial T-DNA into plant genomes (Figure 1.2). The uniqueness of T-DNA genes is that they are of genuine bacterial origin. Nonetheless, they are expressed only in the plant cells as an integrated or autonomous part of plant genome, but are inert in bacterial cells. A feature which is common

and critical to all T-DNAs is the 25-basepair sequence, namely the right (R_B) or left (L_B) border located in each side of the region to be transferred. The T-DNA flanked by two border sequences resides in bacterial Ti (tumor-inducing) or Ri (root-inducing) plasmid in *A. tumefaciens* or *A. rhizogenes*, respectively. When bacteria infect susceptible plants, single stranded T-DNA is synthesized from the right border sequence of the T-DNA and transported into the competent plant cell. To be transferred and integrated into plant genome, the T-DNA has to be kept in transportable conformation (single stranded DNA, or ssDNA), transported through bacterial and plant cell membranes into the host cells and targeted to the nucleus. A group of bacterial *vir* (virulence) gene products function to fulfill these tasks. Transfer of T-DNA into plant cells represents the most obvious example of interkingdom transformation happening in nature.

The border sequences, particularly the R_B , is primarily important in directing T-DNA synthesis. The region between the borders plays no role in transformation. Genetically engineered (disarmed) T-DNA void of pathogenic and opine synthase genes has been explored (McInnes *et al.*, 1989) as a vehicle for routine delivery of genes of interest into plants (Holsters *et al.*, 1978; de Block, 1993; Zambryski, 1992; Zupan and Zambryski, 1995), and as mutagenic tools to discover important genes in plant growth and development (Simon *et al.*, 1983; Sundaresan *et al.*, 1995; Long *et al.*, 1993).

Agrobacterium-mediated gene transfer provides several advantages compared with other methods, in which multi-copy integration, chromosomal rearrangement associated with transformation and integration, and instability of transferred DNA are commonly observed (Walden and Wingender, 1995). Usually only one copy of T-DNA is integrated. DNA or chromosomal rearrangement is rarely reported. Because of the integration, T-DNA is stable and can be passed to offspring through reproduction or somatic propagation from transformed cells. For this reason *agrobacteria* have been intensively studied and *Agrobacterium*-mediated transformations have been widely employed in plant molecular biology/genetic research. This technique was recently extended further from transformation of dicotyledonous to transformation of monocotyledonous plants, such as rice and corn (Nester *et al.*, 1997; Christou, 1996; Ishida *et al.*, 1996), which are not natural hosts of *Agrobacterium*.

A. RHIZOGENES ROL GENES

T-DNA contains genes necessary for bacterial growth (opine metabolism) and pathogenesis in plants. The first category contains genes for biosynthesis of opine compounds using plant metabolites as carbon source for the bacteria. *Agrobacterium* strains are classified based on the type of opines they synthesize and utilize. The second group consists of genes whose products modify normal cellular activities. In *A. tumefaciens* T-DNA, the pathogenicity genes are auxin synthesis genes (*iaaM* and *iaaH*) and a cytokinin synthesis gene (*iptZ*) which are responsible for the tumor (crown gall) syndrome (Zambryski, *et al.*, 1989). Equivalent to the functions of auxin and cytokinin synthesis genes in Ti plasmids, which cause disturbance of plant hormones in infected tissues, there are *rol* genes in *A. rhizogenes* Ri plasmids. These genes are the cause of alteration in root development and morphology. Extensive roots (hairy roots) emerge from the sites of bacterial infection, such as hypocotyl, cotyledon attachment site and cotyledons (White *et al.*, 1985).

The *A. rhizogenes rol* genes have also been found to increase the sensitivity of cells to plant hormones, especially, auxin (Dehio *et al.*, 1993a; Walden *et al.*, 1993; Schmülling *et al.*, 1993a). Tobacco protoplast cells transformed with *rolB* gene were 10,000 times more sensitive to IAA or NAA than untransformed control cells (Maurel *et al.*, 1991a). Epitopical expression of individual or combination of *rol* genes resulted in abnormal phenotypes in shoots, flowers and leaves (Spena *et al.*, 1987; Schmülling *et al.*, 1988, 1989; 1993b, Dehio and Schell, 1993; Kuriota *et al.*, 1992; Nilsson *et al.*, 1996; Maurel *et al.*, 1991b). Although the mode of action of *rol* genes remains unknown, a recent article reported that the ROLB protein is a tyrosine phosphatase (Filippini *et al.*, 1996), indicating the possible participation of *rolB* product in signal transduction pathways. Reminiscent of this finding is the identification of *Arabidopsis rcn1* gene (Garbers *et al.*, 1996). RCN1 is a subunit of protein phosphatase 2A (PP2A-A) and affects seedling responses to auxin transport inhibitor NPA. Signal perception and transduction are commonly through phosphorylation and dephosphorylation of proteins including transcription factors. Together with an earlier finding that ROLB increases the salt-washable auxin binding activity in the plasma membrane (Filippini *et al.*, 1995), it was proposed that the *rolB* gene might play a role in auxin-involved signal transduction (Filippini *et al.*, 1996). The ROLC protein was recently revealed to be associated with a beta-glucosidase activity (Faiss *et al.*,

1996) and postulated that it might be involved in regulation of cytokinin action via cytokinin conjugates. All these discoveries strengthen an early belief that hairy root induction is the consequence of altered hormone balance in root cells (Clare, 1990; Katavic and Jelaska, 1991; Hopkins and Durbin, 1971).

Both *A. tumefaciens* and *A. rhizogenes* can, in the presence of virulence (*vir*) genes, transfer T-DNA into plant cell. Both, when used as transformation vectors, have their advantages and limitations. *A. tumefaciens* is widely used in plant transformation. Standardized protocols are available for different species. A high virulence strain LBA 4404 has been shown to transform both dicots and monocots in high efficiency (Ishida *et al.*, 1996; Hiei *et al.*, 1997). Transformation can be achieved by point inoculation with needles, co-cultivation or vacuum infiltration (Feldmann, 1992). *A. tumefaciens* causes amorphous callus-like tissue which is chimeric in terms of transgenicity. By removal of pathogenic genes from the T-DNA of Ti plasmid, callus-like tissues can be reduced or minimized. Cells and tissues transformed with this method are usually regenerated to whole plants. In contrast, *A. rhizogenes* is used to date only in dicots. To induce hairy roots, intact plants (seedlings) are required. Transformation usually is performed only on some types of tissues, such as hypocotyls, roots, or cotyledons. However, the uniformity of transformation in hairy roots enables quick transgene assays and molecular biological studies for plants which are difficult to regenerate (for example, soybean). Because hairy root formation is a consequence of concerted action of *rol* genes (*rolA*, *B*, *C* and *D*), these genes are preserved in *A. rhizogenes*-mediated root transformation. In recent years, heterologous systems with *A. tumefaciens* harboring Ri plasmid is also being used to induce hairy roots in different plants (Stiller *et al.*, 1997).

A. RHIZOGENES-MEDIATED ROOT TRANSFORMATION TO STUDY NODULATION IN LEGUMES

The *A. rhizogenes*-mediated transformation, though less commonly employed than that of *A. tumefaciens*, has certain desired features. Hairy roots induced by *A. rhizogenes* are highly organized organs. Each hairy root originates from a single transformed cell and therefore is entirely transgenic. Low copy number and stable integration of transgenes are favorable for molecular genetic analysis. All these make the *A. rhizogenes*-mediated transformation a good system to investigate many root-related events (Stougaard *et al.*, 1987), especially for areas where protocols for routine regeneration of transgenic plants

(such as soybean) are still not available. Furthermore, hairy roots grow reasonably fast. Regulation of gene expression and organ development events can be analyzed soon after hairy root emergence. For these reasons, hairy roots have been used in studies of plant root development (Chriqui *et al.*, 1996; Rolfe and McIver, 1996), cytoplasmic male sterility (Vedel *et al.*, 1994), hormone responses (Lincoln *et al.*, 1992), nutrient uptake (Hoflich and Kuhn, 1996) and metabolism (Hamill *et al.*, 1994), and bacterial RNA (*rolA*) splicing in plants (Magrelli *et al.*, 1994). In legume nodulation research, this system has been widely employed (Beach and Gresshoff, 1988; Hansen *et al.*, 1989). Transformation with *A. rhizogenes* strains has been reported in soybean *Glycine max* (Cheon *et al.*, 1993;), alfalfa (Frugis *et al.*, 1995; Novikova and Pavlova, 1993), common bean *Phaseolus vulgaris* (Carsolio *et al.*, 1994; Hamill *et al.*, 1987), pea *Pisum sativum* (Vijn *et al.*, 1995), *Medicago truncatula* (Thomas *et al.*, 1992), *Lotus japonicus* (Stiller *et al.*, 1997), and many other legume plants (Perlick *et al.*, 1996; Suzuki *et al.*, 1992; Stiller *et al.*, 1992). In our laboratory we have expanded and successfully used this method to study nodulation in soybean (Bond, 1993, Bond *et al.*, 1998) and *L. japonicus* (Stiller *et al.*, 1997; Chian *et al.*, 1998).

Soybean transformation

Soybean was one of the major crop subjected to extensive study of symbiotic legume-*Rhizobium* interactions. It was also among the plants in which successful transformation was originally reported. Particle bombardment of immature embryonic axes and regeneration of transgenic plants from cell/tissue culture has since been used as a *quasi* standard for this plant (McCabe *et al.*, 1988; Christou, 1996). This success was soon followed by agroinfection of cotyledon explants (Hinchee *et al.*, 1998). 'Roundup Ready' herbicide-resistant soybean (Monsanto, St. Louis, MO) had been sold commercially and has become a flagship in the genetic engineering of agriculturally important crops. Meanwhile transgenic soybean resistant to the wide-spectrum herbicide atrazine is on field trial (Yue *et al.*, 1990). Transgenic soybean lines carrying foreign genes for increased protein content (Nodlee *et al.*, 1996), *Bacillus thuringiensis* toxin for insect resistance (Steward *et al.*, 1996), plant virus coat protein for disease resistance (Di *et al.*, 1996) and chimeric reporter gene CaMV 35S-GUS (Yang and Christou, 1990; Christou and McCabe, 1992; Zhou and Atherly, 1990) have also been reported. Despite these successes, the vast investment in molecular genetic engineering of this crop during the last decade has turned

out to be less productive than expected. Soybean remains among the species highly difficult to manipulate (Finer *et al.*, 1996; Chee and Slightom, 1995; Luo *et al.*, 1994). Transformation frequency was shown to be heavily cultivar-dependent (Dhir *et al.*, 1992; Christou and McCabe, 1992; Christou, 1990). The complication is exaggerated when regeneration of transgenic plants becomes a necessity (Luo *et al.*, 1994). Tissues susceptible to transformation usually are incompetent for regeneration (Christou and McCabe, 1992; de Block, 1993; Yang and Christou, 1990; Chee and Slightom, 1995). Thus only a few types of tissues, such as embryogenic tissues and cotyledonary node, are commonly used for this purpose (Finer *et al.*, 1996; Dhir *et al.*, 1992).

Bond (1993) developed an *A. rhizogenes*-based soybean root transformation system for nodulation research. Up to 90 % of 'Peking' plants can be transformed by strain K599 and its derivatives to produce hairy roots. Transgenic nodules have been harvested from these plants. However, this system is cultivar-dependent. When other genotypes of soybean were used, the transformation frequency was lower. It takes four weeks for hairy roots to emerge from 'Peking' and even longer from 'Bragg' and its supernodulation mutant. Usually, fully developed nodules can only be obtained about three months after seed germination. Because regeneration of transgenic plants from hairy roots is still not possible up to now, continuous genetic and molecular biological assay using the same transgene line cannot be performed with this system. Nevertheless, as far as regeneration has not become routine practice, the K599-mediated root transformation method remains to be a reasonably sound choice for the study of root-related events, such as nodulation in soybean.

Looking for model legumes

In recent years, two small weeds, *Lotus japonicus* and *Medicago truncatula*, have been proposed as model legumes for study of symbiotic nitrogen fixation (Handberg *et al.*, 1992; Jiang and Gresshoff, 1993; Barker *et al.*, 1990). These plants have many desirable features for molecular genetic research: fast growth rate and short life cycle, flexible growth conditions, autogamous, small diploid genome with limited number of chromosomes and susceptible to various transformation and regeneration techniques. The two plants also represent the two major nodulation types of legumes. *L. japonicus*, like soybean, initiates nodulation through subepidermal cell division (K. Szczyglowski, personal communication)

and produces spherical, determinate nodules. *M. truncatula*, on the other hand, has typical cylindrical indeterminate nodules resembling that of alfalfa.

L. japonicus, a relative of birdsfoot trefoil, is also a perennial forage legume. The life cycle (from seed to seed) takes about three months. The wide geographic distribution indicates its flexibility for growth conditions. Because of the small genome (about 350 - 400 mega base pairs per haploid genome) and small number of chromosomes ($2n = 6$), it is especially suitable for molecular manipulations. Determinate type (spherical) nodules are produced when inoculated with either fast growing *Rhizobium loti* or slow growing *Bradyrhizobium* strains (Stougaard and Beuselinck, 1996). Susceptibility of *L. japonicus* to *A. tumefaciens* transformation has been shown by different laboratories (Handberg *et al.*, 1992; Jorgensen *et al.*, 1994; Stiller *et al.*, 1997). Transgenic plants from *A. tumefaciens* transformed hypocotyl are obtained in about six to eight months (Stiller *et al.*, 1997; Handberg *et al.*, 1994).

The rapidly increasing interest in *L. japonicus* as a model legume is demonstrated by the significant growth of publications in the last two years and the voluminous work in diverse areas: functional genomics (Kondorosi *et al.*, 1997; Pillai *et al.*, 1996), signal transduction pathways (Borg *et al.*, 1997; Poulsen *et al.*, 1994), plant responses to hormones (Kawaguchi *et al.*, 1996), new approaches to identify nodulation-specific genes and nodulation mutants (Thykjaer *et al.*, 1997; Thykjaer *et al.*, 1995), biochemical and immunological studies of genes involved in metabolism of nitrogenous compounds (Waterhouse *et al.*, 1996; Tajima *et al.*, 1996), searching and developing more efficient transformation techniques (Oger *et al.*, 1996; Jorgensen *et al.*, 1994) and identifying nodulation-specific genes (Kapranov *et al.*, 1997; Szczygłowski *et al.*, 1997; Schauser *et al.*, 1995).

In our laboratory, we further expanded the potential of this plant for *A. rhizogenes*-mediated root transformation and developed alternative regeneration protocols (Stiller *et al.*, 1997; Chian *et al.*, 1998). T-DNA insertional mutagenesis based on the new protocol for tagging genes important in nodulation and root development has detected several potential root lines (J. Stiller, personal communication). Conventional sexual crossing within the genus has been a routine practice and recombinant inbred *L. japonicus* lines have been produced (Jiang and Gresshoff 1997). Establishment of genetic markers and genome

mapping is in progress (Jiang and Gresshoff, 1997). Hopefully, all these efforts as well as those from other laboratories will pool together to contribute towards an understanding of symbiotic nitrogen fixation and organ development in plant roots.

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Part II

**SOYBEAN ROOT TRANSFORMATION WITH
AGROBACTERIUM RHIZOGENES STRAIN K599 AND
ITS DERIVATIVE K599 GUS-I**

Abstract

An *Agrobacterium rhizogenes* strain K599-based soybean root transformation system was developed in this laboratory. This system showed high frequency of hairy root induction and can be used as alternative to study gene regulation during nodulation in soybean, which is still difficult to obtain transgenic plants. The repeatability and consistency of this high frequency transformation protocol was tested in this study. Modifications were made to optimize growth of hairy roots and whole plants and to simplify media and the protocol. In most experiments, hairy roots were induced from 70 to 90 % soybean plants, confirming that this is a reliable method when cultivar 'Peking' is used. In addition, meristematic nodules were obtained from transgenic roots of 'Peking' as well as 'Bragg'. The GUS-positive abnormal nodules observed indicates that the K599 derivative strain carrying the p35S GUS-Int binary vector is capable of altering nodule phenotype. Acetylene reduction experiments showed that some of the abnormal nodules retained low nitrogenase activity.

Introduction

The high oil and protein content of soybean has made it one of the most important agricultural crops in the world. Besides the nutritional value, the ability of soybean and other legume plants to symbiotically fix atmospheric nitrogen into plant-usable ammonia also makes it an essential part of integrated crop management for soil amendment and yield improvement in developing countries. In developed countries such practice is less needed because of the lower cost and availability of chemical fertilizers. However, the relationship between soybean and nitrogen-fixing bacteria *Bradyrhizobium* species and the formation of a specific structure, the root nodule, have brought about a vast academic interest in areas of plant-microbe interaction and plant organ development.

Nodule formation is commonly the consequence of legume-*Rhizobium*/*Bradyrhizobium* interaction. This interaction is specific. Different species of legumes nodulate only in response to certain species of *Rhizobium*/*Bradyrhizobium*. Normally, legume plants will not form nodules in the absence of compatible rhizobia. The requirement of rhizobia for the induction of nodule organogenesis is attributed to secreted lipo-

oligosaccharide Nod factors, the products of bacterial nodulation genes (Roche *et al.*, 1996a; Schultze *et al.*, 1995; Staehelin *et al.*, 1994; Vijn *et al.*, 1993). Decorations in the reducing and non-reducing end sugars of the oligochitin backbone determine the host specificity of Nod factors (Freiberg *et al.*, 1997; Schultze *et al.*, 1992; Roche *et al.*, 1991; Fisher *et al.*, 1993). However, study in spontaneous nodulation variants of alfalfa (Nar⁺, or nodulation in the absence of *Rhizobium*. Truchet *et al.*, 1988; Caetano-Anollés *et al.*, 1991) indicated that nodule development *per se* is entirely controlled by plant genes (Caetano-Anollés and Gresshoff, 1991; Gresshoff, 1993). The Nar⁺ lines give rise to normal-looking nodules without rhizobial infection. Electron microscopic examinations exhibited that these empty nodules have the same organization of *Rhizobium*-induced functional ones (Joshi *et al.*, 1991). This notion was further supported by induction of empty nodules with purified or synthetic Nod factors (Freiberg *et al.*, 1997; Long, 1996; Roche *et al.*, 1996b; Horvath *et al.*, 1993; Stokkermans *et al.*, 1995). Once started, nodule organogenesis follows certain patterns and has distinct fates, thus is a programmed process. This is evident in the two major types of nodules. In tropical legumes such as soybean, French bean and *Lotus japonicus*, nodule meristematic activity ceases earlier. Nodules formed in these plants are spherical (determinate nodules). In contrast, nodules in temperate legumes such as alfalfa, pea, vetch and *Medicago truncatula* are cylindrical (indeterminate nodules) due to persistent nodule meristematic activity. The distinction of nodule types between tropical and temperate legumes is not because they are inoculated by different rhizobial species. *Bradyrhizobium* strains ANU289 and CP283 infect both the non-legume *Parasponia* and the forage legume siratro (*Macroptilium atropurpureum*). The same bacterium induces determinate nodules on siratro but indeterminate coralloid nodules on *Parasponia* (Price *et al.*, 1984; Scott and Bender, 1990). Nodule induction with heterologous Nod factors provides further evidence that the morphology of empty nodules is determined by host legumes but not the Nod factors (Orgambide *et al.*, 1995; Roche *et al.*, 1996a; Stokkermans *et al.*, 1995)

To dissect the elements controlling nodule development, methods have to be established to study the functions of genetic components. Transformation is the cornerstone for such approach and is now routinely used. Isolated genes are manipulated and then “delivered” into organisms or cells in order to study their functions and the effects of gene modifications. New genes are also identified by transformation-based insertional mutagenesis (T-DNA or T-DNA mediated transposon tagging). Such methods have

advantage over conventional chemical and irradiation mutagenesis in that it is quick and easy to identify and isolate genes causing altered phenotypes (Koncz *et al.*, 1996; Fritze and Walden, 1995).

Soybean was among the first plants subjected to molecular engineering. Transgenic plants were obtained by particle bombardment of the immature embryogenic axis (McCabe *et al.*, 1988; Christou, 1996). This success was soon followed by agroinfection of cotyledon explants with disarmed *A. tumefaciens* strain A208 (Hinchee *et al.*, 1988). 'Roundup Ready' soybean resistant to non-selective herbicide glyphosate (Monsanto, St. Louis, MO) is now a dominant soybean line in seed markets. Transgenic plants with the insect resistance BT toxin gene (Steward *et al.*, 1996; Parrot *et al.*, 1992) and increased storage protein content (Nodlee *et al.*, 1996) have been reported as well. Attempts were also aimed at genetic engineering to express the virus coat protein for cross protection of viral diseases (Di *et al.*, 1996) and to develop resistant cultivars against soybean cyst nematode. Manipulation of the composition of protein content (Townsend *et al.*, 1992) and unsaturated fatty acid contents is a "hot spot" of academic research and commercial application (G-H. Miao, personal communication). Despite these successes, investment on molecular genetic engineering of this crop has been less productive. Studies from different laboratories revealed that soybean is much more difficult to manipulate than was expected earlier. Transformation and, particularly regeneration, are highly cultivar-specific and tissue-type-dependent (Finer *et al.*, 1996; Chee and Slightom, 1995; Dhir *et al.*, 1992).

Agrobacterium rhizogenes infection of plants results in the formation of transformed roots, the hairy roots. The hairy roots have been explored as a short-cut to study root related events such as nodulation (Stougaard *et al.*, 1987; Cheon, *et al.*, 1993). The advantages of this technique are: (i) hairy root induction and growth are fast; (ii) low copy number of T-DNA integration; and (iii) stable transgene integration. And more importantly, because hairy roots are entirely transgenic, tedious selection and regeneration processes for transgenic plants from chimeric tissue such as crown gall (induced by *A. tumefaciens*) can be by-passed.

Although soybean transformation with both *A. tumefaciens* and *A. rhizogenes* strains have been demonstrated, the success and efficiency of transformation depends on both soybean genotype and bacterial strains (Owens and Cress, 1985; Bryne *et al.*, 1987).

Attempt to increase transformation efficiency by adding acetosyringone during infection of soybean with *A. rhizogenes* was made (see Bond *et al.*, 1998, also in this study). Acetosyringone, which has been shown to be the key factor for the success in *Agrobacterium*-mediated monocotyledonous plant (rice and maize) transformation (Ishida *et al.*, 1996; Hiei *et al.*, 1997), did not increase tumorigenicity in soybean. Steve Farrand's group (Urbana, IL) screened several *A. rhizogenes* strains of different opine types. They showed that one cucumopine type strain, K599, has relatively high virulence on most soybean genotypes tested; cultivar Peking was considerably susceptible to this strain (Savka *et al.*, 1990). This work opened the door for efficient soybean root transformation.

Our laboratory started to develop a K599-based soybean root transformation system for nodulation research in 1989. Higher transformation frequency was achieved. The observation of morphologically altered nodules on *A. rhizogenes*-induced hairy roots by Bond *et al.* (1993; 1998) provided the first insight into foreign genes expressed in plant cells, that might be able to change the "destiny" of programmed organ development. These abnormal nodules are carrot-shaped or multi-lobed/branched. Ultrastructure study of such nodules by electron microscopy revealed long lasting (compared to normal soybean nodules) meristem tissues on the distal region. Because no such abnormal nodule was observed in wild-type soybean roots, it is most likely that they resulted from the interference of bacterial genes with nodule morphogenesis. The *A. rhizogenes rol* genes (*rol A, B, C* and *D*) that affect perception and sensitivity of cells to plant hormones are the primary candidates for causing such changes. The objective of this study is to refine the *A. rhizogenes*-mediated transformation system and test the repeatability of the results obtained by Bond (1993).

Materials and Methods

BACTERIAL CULTURE

Agrobacterium strains used in this study are listed in Table 2.1. About 40 milliliter (mL) liquid Modified Bergersen Medium (BMM, 1976) was inoculated with test bacterial strains and incubated at 28 °C under constant agitation (150 rpm) for 3 days. Prior to infecting plants, bacteria were pelleted at 4,000 rpm and resuspended in 1 mL fresh BMM. The estimated bacterial density was 10^9 cfu mL⁻¹. In treatments where acetosyringone was

applied, the chemical was added to the bacterial culture at a final concentration of 200 μM . Approximately 1 μL of bacterial suspension was used to infect each plant.

Table 2.1. *Agrobacterium* strains tested in this study.

strain (plasmid)	name	opine type	antibiotic resistance	source
<i>A. tum.</i> C58C1 (pRi15834::GUS)	AR12	Agropine	Rif ^r , Kan ^r	J. Stougaard
<i>A. tum.</i> C58C1 (pRi15834::cat)	AR14	Agropine	Rif ^r , Kan ^r	J. Stougaard
<i>A. rhiz.</i> 15834 (pRi15834 a, b, c)	15834	Agropine	wild-type	J. Stougaard
<i>A. rhiz.</i> A4 (pRiA4 a, b, c)	A4	Agropine	wild-type	E. Nester
<i>A. rhiz.</i> R1000 (pRiA4b)	Arqual	Agropine	Sm ^r	J. Stougaard
<i>A. rhiz.</i> A4 (pRiA4 a, b, c)	A4RSII	Agropine	Rif ^r , Sp ^r	D. Tepfer
<i>A. rhiz.</i> 1855 (pRi1855)	LBA9402	Agropine	Rif ^r	D. Tepfer
<i>A. rhiz.</i> 8186	8196	Mannopine	wild-type	S. Farrand
<i>A. rhiz.</i> K599	K599	Cucumopine	wild-type	S. Farrand
<i>A. rhiz.</i> K599 (p35SGUS-I)	K599GUS-I	Cucumopine	Kan ^r	J. Bond

Bradyrhizobium japonicum USDA110 was used to nodulate soybean. Bacteria were grown in liquid modified YEM medium (Bhuvaneswari *et al.*, 1983). After 3-4 days culture in 28 °C with constant agitation (150 rpm) the bacterial suspension was used directly as inoculum. Approximately 10⁸ cfu were applied to each plant.

A. RHIZOGENES-MEDIATED ROOT TRANSFORMATION PROCEDURES

Soybean cultivars Peking, Bragg and the supernodulation mutant nts382 (derived from Bragg) were used in this study. Several methods for surface sterilization of soybean seeds were tested (Table 2.2). After the final wash, seeds were coated with fungicide Banrot and CaptanTM at a concentration of 100 $\mu\text{g mL}^{-1}$. All steps thereafter were performed under aseptic conditions. Soybean seeds were germinated on 1/10 Gamborg's B5 plates (B5 7264, Sigma, St. Louis, MO) supplied with 2% sucrose and 1.0% Difco Bacto agar, pH 5.8, or simply on wet filter paper, at room temperature (22-25 °C) in the dark for 3 days.

Table 2.2. Methods of soybean seed surface sterilization

Treatment	Concentration (%)	Time (minutes)	Wash (x) with sterile water
Sodium Chlorite (w/v)	1	5	5
Ethanol (v/v)	70	10	5
Mercury chloride (w/v)	0.1	5	5
Ethanol + H ₂ O ₂ (v/v)	70 % + 3 %	10	5

Seedlings about 3-4 cm long with cotyledon unopened were lightly wounded at the hypocotyl region with a surgical scalpel or a 26 gauge needle. Approximately one μL agrobacterial suspension (10^{10} cfu mL^{-1}) was applied immediately after wounding. Two inoculated seedlings were grown in a sterile, clear Magenta jar (10 x 7 x 7 cm³, Magenta Corp, Chicago, IL) containing 50 mL of 1/5 B5 medium with 2% sucrose and 0.85 % agar). The roots of seedlings were inserted into the agar to keep plants in an upright position. Caution was taken to avoid direct contact of the inoculation site with the medium, when inserting the root into the agar. The Magenta jars with inoculated seedlings were kept at room temperature in the dark for 2 days to facilitate T-DNA transfer, before moving into a growth chamber with a day-night temperature/light regime of 30 °C/16 hrs. - 22 °C/8 hrs.

Mosaic soybean plants with transgenic hairy roots were generated in 2 to 8 weeks after inoculation, depending on the combination of the cultivar and inoculum. When the hairy roots emerged from the inoculation (wound) site were 1-2 cm long, plants were taken from the Magenta jars, the untransformed tap roots were removed and plants were then transferred to new Magenta jars with liquid 1/5 - 1/4 B5, or Mon Mor medium (MM medium. Savka *et al.*, 1990) supplemented with 3% sucrose. Selection of transgenic roots with kanamycin resistance was done by using the medium in the presence of 100 $\mu\text{g}\text{mL}^{-1}$ kanamycin. To kill agrobacteria in hairy roots, 500 $\mu\text{g}\text{mL}^{-1}$ carbenicillin, 100 $\mu\text{g}\text{mL}^{-1}$ cefotaxime (claforan), 75 $\mu\text{g}\text{mL}^{-1}$ amoxicillin, 37.5 $\mu\text{g}\text{mL}^{-1}$ clavulanic acid were added to the MM medium, while in 1/5 or 1/4 B5 only carbenicillin was used. Mosaic plants were grown in the new media for 2 weeks to allow adequate root growth. The transgenic status of roots was examined by Southern blotting, polymerase chain reaction (PCR) and GUS

assay. Plants with positive transgenic roots determined by visual examination, or any one or more of the above techniques were transferred to soil in the greenhouse.

GUS ASSAY

Assay for β -glucuronidase activity in transgenic roots carrying the *uidA* (Blanco *et al.*, 1985) or *uidA*-intron gene (Vancanneyt *et al.*, 1990) was performed according to Jefferson (1987). One piece of root about 1 cm long was cut and immersed in GUS assay buffer (50 mM potassium phosphate, pH 7.0; 1 mM EDTA; 1 mM ferrocyanide; 1 mM ferricyanide; 0.1% Triton X-100; 5 mg X-glucuronic acid per 20 mL solution) in a Falcon 24-well plate (Becton Dickinson, New Jersey). The plate was then sealed with tape and incubated at 37 °C overnight. β -glucuronidase activity was determined under a stereoscope for the blue color.

SOUTHERN BLOTTING ANALYSIS

To confirm the transgenic nature of hairy roots, total DNA was isolated from soybean roots according to the protocol by Dellaporta *et al.* (1983). A modification aimed to reduce the polysaccharide content in DNA preparation (Li *et al.*, 1994) was adopted. In Southern hybridization, 6-15 μ g DNA of each sample was digested with restriction endonucleases and run in a 0.9% agarose gel. The gel was then stained with 2 μ g mL⁻¹ ethidium bromide solution for 15 min. and visualized under UV light. DNA was transferred from agarose gel onto Zeta-Probe (Bio-Rad) Nylon membrane using vacuum blotting (LKB) and immobilized by UV in a cross-linker oven (Stratalink, Stratagene). *A. rhizogenes* T-DNA genes *rolB*, *rolC* and the *E. coli uidA* gene fragment were used as probes to detect transformation. Random Primer Labeling kit (Boehringer-Mannheim) and Megaprime DNA Labeling Kit (Amersham) were used to label the DNA probes with radioactive α -³²P, following the manufacturer's instructions. The stringency of hybridization of probe to the membrane and the following washes were varied from 60 - 67 °C, taking into account of the DNA quantity loaded on gel, and sequence homology between the probe and the transformed genes.

PCR

The same DNA samples for Southern blotting were also used in PCR. Primers for *A. rhizogenes rolB* (forward: 5' CTATTCCTTCCACGATTTC AACC 3', reverse: 5' CTCCAGCAAGTGAATGAACAAGG 3'), and *rolC* (forward: 5' TCGATGGATATTGACGAAGAAGG 3', reverse: 5' TGCACTCGCCATGCCTCACCAAC 3') were designed using Oligo software (National Biosciences, Plymouth, MN)). A final concentration of 50 μ M each dNTP (dATP, dCTP, dGTP and dTTP), 0.1-0.5 μ M each primer, 2 mM MgCl₂ and 1 unit Amplitaq DNA polymerase (Perkin-Elmer) were added to 1X reaction buffer containing 100-200 ng total DNA or 4 ng plasmid DNA in a total 50 μ L volume. The *rol* gene containing plasmids pPCV002-ABC and pPCV002-B1000 were kindly provided by Dr. A. Spena (Max-Plank-Institut für Züchtungsforschung, Köln, Germany) and were used as positive control. The internal control for soybean genomic DNA was obtained using a pair of primers designed in this study for the soybean cyclin B gene.

Results

SEED SURFACE STERILIZATION

Previously, soybean seeds were surface sterilized with 2 % sodium hypochlorite (about 40 % Clorox). The detrimental effect of the chemical was apparent. Although the germination rate in the treatment was comparable to the untreated control (80 - 90 %), bending of roots was observed in majority of germinating seeds. By reducing the concentration of sodium chlorite (0.5 - 1.0 %), the toxicity of chemical to cultivar Peking can be minimized, as most seeds had straight roots. However, the harm to cultivar Bragg and its nodulation mutants nts382 and nod49, nod139 could not be substantially alleviated. Severe distortion of gravitropism was observed in some seedlings transferred to pots. These plants showed a upside down growth pattern with roots growing upwards and exposed to air, while cotyledons buried permanently in soil. Distortions of root growth by chemical toxicity was greatly reduced in treatments with either 0.1 % mercury chlorite, 70 % ethanol alone, or 70 % ethanol plus 3 % H₂O₂, as measured by root appearance in germinating seedlings. Germination rate was improved or unchanged without compromising sterility.

MODIFICATION OF THE PROTOCOL FOR MAKING *A. RHIZOGENES*-INDUCED CHIMERIC PLANTS

The basic protocol developed by Bond (1993), who worked as a graduate student in this laboratory, was utilized in this study. Several modifications were made in order to improve the growth of roots, and plant as a whole, and to simplify the procedure. The 1/10 B5 medium for hairy root induction after *Agrobacterium* infection used by Bond (1993) was obviously too weak to support seedling growth for 4 - 8 weeks. Soybean grown on the medium in Magenta jars usually turned yellowish after 2 weeks. Increase in the medium strength helped plants staying green for longer time. On stronger B5 medium (>1/4 strength), *A. rhizogenes* colonies formed more easily and fast. As a result, seedling growth was suppressed. Excessive bacteria were also difficult to eliminate and caused contamination in liquid culture. The medium strength was finally chosen at 25 % of the normal concentration (1X).

The Mon Mor medium (MM) for promoting hairy root growth and eliminating

contaminating agrobacteria was composed of Monnier's salts (1976) and Morel's vitamins (1951). Precipitation of chemicals was commonly observed, which was clearly harmful to plants. As a consequence, soybean grown in this medium died fast when this happened. In addition, excessive antibiotics used by J. Bond (many of them cannot be obtained through normal purchasing routes for scientific research) made medium preparation complicated. Kanamycin plus claforan or carbenicillin were the original components of Mon Mor medium. However, in comparison experiments, this medium with excessive antibiotics could not demonstrate that it was superior to media without the additional 3 to 4 other antibiotics in suppressing *Agrobacterium* growth and colonization. Several media (B5, MS, Modified Schenk-Hildebrandt, Herridge's and Fahreaus' supplied with nitrogen sources) in different concentrations were tried to replace MM. One quarter strength B5 with 100 $\mu\text{g mL}^{-1}$ kanamycin, and 400 $\mu\text{g mL}^{-1}$ claforan (Cefotaxime) or 500 $\mu\text{g mL}^{-1}$ carbenicillin gave the best results as judged by plant appearance and was used in most of the subsequent experiments.

SOYBEAN CULTIVAR-A. *RHIZOGENES* STRAINS COMPATIBILITY TEST

The efficiency of transformation of soybean by *Agrobacterium* depends on both susceptibility of host cultivars and the virulence/comparability of bacteria. Among 10 *Agrobacterium* strains tested in this study for hairy roots induction, *A. rhizogenes* K599 and its derivative carrying the binary vector (p35SGUS-I) K599::GUS-I gave the highest transformation frequency in Peking and Bragg, including the Bragg supernodulation mutant nts382 (Table 2.3). This was consistent with the results of Savka *et al.* (1990) and Bond (1993).

ABNORMAL NODULES

Bond (1993) observed in his original work that about 10 -15 % Peking plants transformed with K599 or K599 harboring a chalcone synthase gene (CHS)-containing plasmid produced abnormal nodules on their hairy root system. Instead of spherical shape in wild-type nodules, these nodules were elongated or carrot-like shaped. Occasionally nodules were branched and formed 2 or more lobes. Anatomic studies showed that the nodule meristem remained for longer time and could be distinguished even in mature nodules (Bond *et al.*, 1998). Although the GUS activity could be detected from normal-

looking nodules on hairy roots, it was not seen in any meristematic nodules. This result was interpreted as the p35SGUS-I plasmid interfering with the meristematic nodule phenotype, and over-expression of chalcone synthase gene enhancing the Ri-plasmid function in altering nodule morphogenesis (Bond, 1993).

Table 2.3. Infectivity of different *Agrobacterium* strains on soybean cultivar Peking, Bragg and nts382.

	Peking	Percentage of hairy root induced on	
		Bragg	nts382
A4 wt ^a	0	0	0
LBA9402 ^a	0	0	0
15834 ^a	0	0	0
AR10 (AR12, 14) ^a	0	0	0
8196 ^a	0	0	0
ARqual ^a	< 10	0	ND
A4RSII ^a	< 10	0	ND
K599 ^b	70 (50 - 100)	40 (20 - 70)	37 (25 - 60)

The mean rate (and range in parentheses) of hairy root induction was based on 20 plants^a inoculated with strains other than K599, more than 300 plants on Peking and Bragg, or about 100 on nts382 infected with K599^b. ND, not determined.

In my study only wild-type K599 and K599::p35SGUS-I were used. Meristematic nodules were obtained from both Peking and Bragg. Most of them were elongated and cone-shaped. Some were branched and budded out (Figure 2.1A and E). The budding did not always occur from the distal end of nodules. Longitudinal sectioning of branched nodules showed that these branches (Figure 2.1A) had similar organization as in wild-type nodules. They had clearly originated from the same nodule primordium, rather than having generated from a cluster of primordia (Figure 2.1 B). The nodule meristem, nonetheless, may not always have initiated from the sub-epidermal cortical cells giving rise to nodule primordia, as evidenced by the fact that some buds located halfway along the abnormal nodule axis (Figure 2.1E). It appears that, in these nodule buds meristem gained activity at a later stage. Blue-colored and cone-shaped nodules were harvested from Peking hairy roots (Figure 2.1A), indicating that K599::p35SGUS-I was able to trigger alteration in nodule morphogenesis.

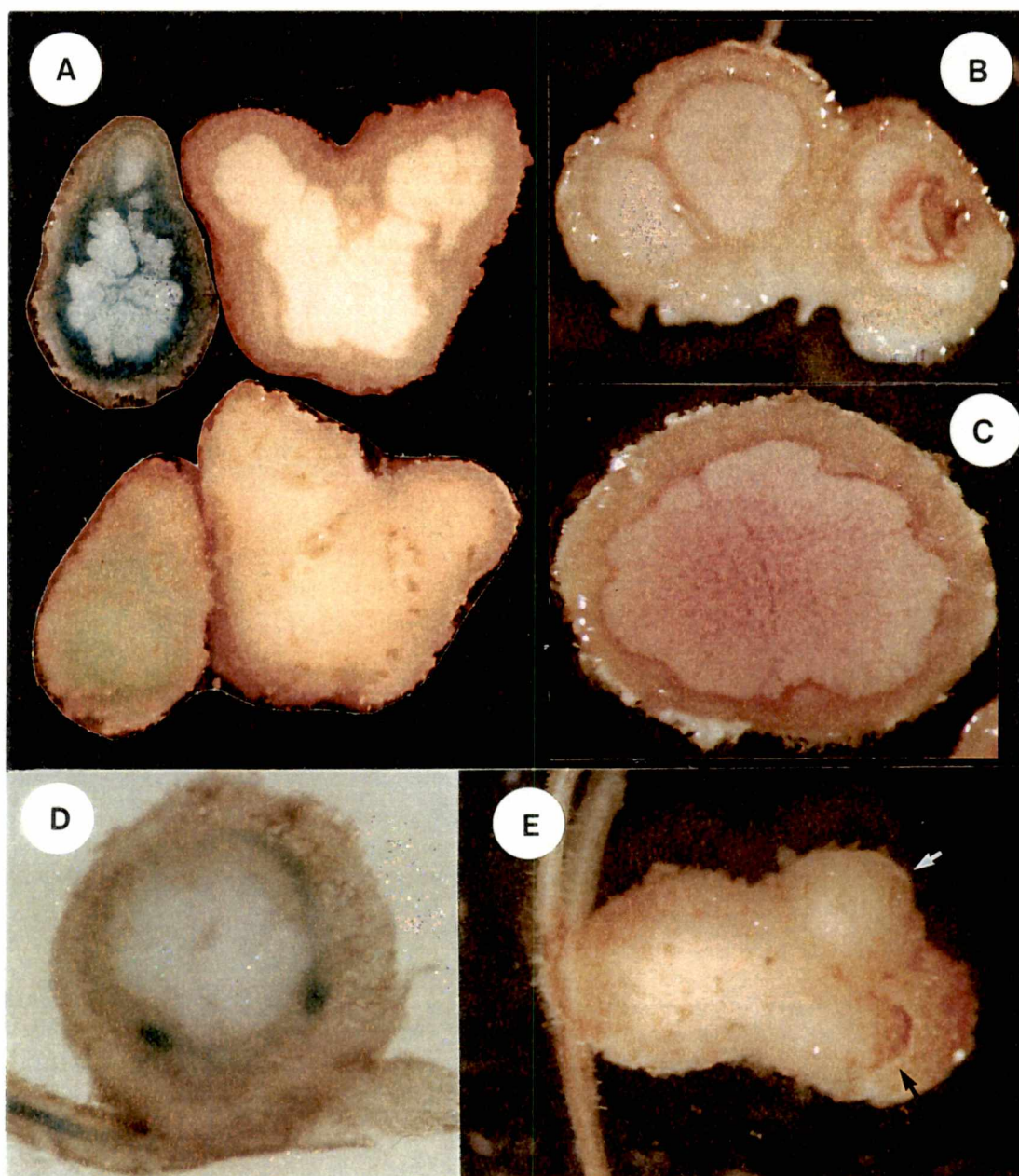


Figure 2.1. Abnormal nodules formed on hairy roots of soybean induced by K599 or K599::p35SGUS-Int strains of *A. rhizogenes*. **A.** cone-shaped or branched nodules from K599::p35SGUS-Int and wild-type K599 transformed hairy roots, respectively. Meristematic tissue can be recognized in the tip of the cone-shaped nodule. The branches, due to a divided meristem, originated from the same nodule primordium (Right, upper panel) are apparently different from clusters of nodules (**B**), which bears clearly boundaries between nodules. No discrete regions as seen in abnormal nodules can be identified inside the wild-type nodules (**B** and **C**). Sometimes, the meristem in abnormal nodules was activated or divided at a later stage, resulting in the formation of buds (indicated by arrows) along the longitudinal axis of the nodule (**E**). Many of the transgenic nodules are impaired in their nitrogen-fixing ability, as indicated by lacking pink color (leghemoglobin) in the infection zone (**A** and **D**).

FUNCTIONALITY OF MERISTEMATIC NODULES

The inability to detect nitrogenase activity in acetylene reduction experiments by Bond (1993) suggested that these abnormal nodules were non-functional. However, the infection thread in the infection zone behind the meristem and the pink color showing putative leghemoglobin protein expression, left the question open. This experiment was repeated in this study. Compared with normal nodules, meristematic nodules reduced substantially lower levels of acetylene (Table 2.4). Some nodules failed to demonstrate nitrogenase activity at all. Thus, these nodules were indeed impaired in their ability to fix atmospheric nitrogen, presumably, because of their altered morphology or early senescence. It would be of value to conduct a time-based developmental study to see whether young nodules are Fix⁺.

Table 2.4. Nitrogenase activity of meristematic nodules

	Acetylene reduction (μ moles acetylene reduced $\text{g}^{-1}\text{hr}^{-1}$) Average/range
Meristematic Nodules	4.1 / 0 - 11
Wild-type Nodules	37.8 / 26 - 42.3

EXAMINATION OF TRANSFORMATION AND INTEGRATION BY SOUTHERN HYBRIDIZATION AND PCR

Several methods were employed to examine the transgenic state of hairy roots. The blue color in some roots and nodules was a very reliable indication of expression of reporter gene *uidA* (Jefferson, 1987) in plants. The p35SGUS-I was an engineered plasmid. To prevent a false signal produced by the reporter gene (in plasmid) carried by the bacteria in plant tissue, a plant intron was inserted into the *E. coli uidA* gene (Vancanneyt *et al.*, 1990) encoding the β -glucuronidase. In control experiments, it was shown that K599 carrying the p35SGUS-I plasmid was not able to break down the substrate of *uidA* product 5-bromo-4-chloro-3-indolyl-beta-D-glucuronic acid (X-Gluc) *in vitro*. The blue color in transformed plants can only be attributed to plant cellular expression of the active enzyme obtained after splicing of the intron from the transcript of the transgene.

Although it has been reported that binary vectors co-transform with Ri- or Ti-plasmids in high frequency, a positive result from GUS assay does not prove the root is a hairy root. Likewise, a negative result in a GUS assay cannot be used to exclude a plant as non-transgenic. Southern hybridization was performed as a supporting and alternative method to check transformation, when GUS-negative results were obtained. In these experiments the GUS gene and *rolC* gene from pPCV002-C plasmid (Schmülling *et al.*, 1988) or PCR amplified *rolC* fragment of *A. rhizogenes* strain A4 (agropine type) were used as probe. In one experiment containing 8 samples (3 from Bragg and 5 from Peking), the GUS probe detected a single band from 7 samples and 3 bands from one sample (data not shown). This result was in agreement with reports that *Agrobacterium*-mediated transformation usually has high co-transformation frequency of T-DNA with binary vectors (Hamill *et al.*, 1987) and low copy-number of integration (Christou, 1996; de Block, 1993). It was estimated by Southern blotting that about 70 % hairy roots in chimeric transgenic soybean were single copy for the GUS gene. With A4 *rolC* gene as probe, one or two homologous sequences were detected in transformed Peking, Bragg and nts382 samples digested with *HindIII*, but not in wild-type controls (Figure 2.2A). The same size of fragment hybridized to *rolC* probe in samples from different soybean genotypes could have resulted from the internal *HindIII* sites of cucumopine-type T-DNA. In pRi1724 (Mikimopine-type) T-DNA, this enzyme generates a 8 kb fragment, while in A4 strain (agropine-type), its cleavage of TL-DNA produces 5 - 6 fragments ranging from 0.2 to 3.5 kb. Because the complete T-DNA sequence of cucumopine plasmid is not available, the explanation of internal restriction sites cannot be confirmed.

In a separate experiment, the GUS-intron probe hybridized to a single band from an *EcoRI* digest and 3 bands from a *HindIII* digest of Peking sample (Figure 2.2B). Neither the *HindIII* nor the *EcoRI* site is resided in the *uidA* gene; nevertheless, the 2.9 kb signal is close to the predicted size of p35SGUS-Int fragment digested with *HindIII* (2.93 kb). The bands detected in *EcoRI* digest could be an indication of more than one copy of transforming DNA.

The transgenic status of hairy roots induced by K599 and its derivative was also examined by PCR. A pair of primers designed for A4 *rolB* amplified a fragment from both the K599 cell lysate and K599::p35SGUS-I transformed Peking roots with the expected size (500 bp). No amplification occurred in untransformed control samples (Figure 2.2C).

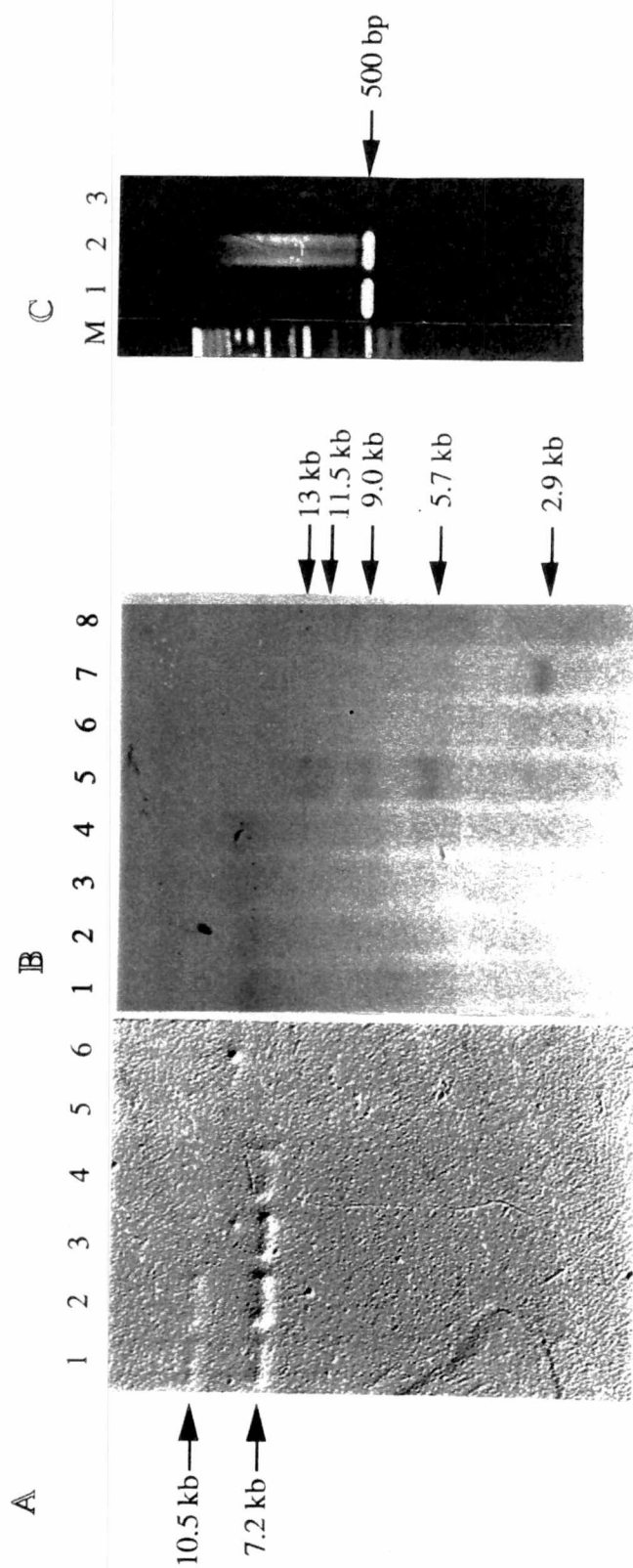


Figure 2.2. Transgenic soybean roots confirmed by Southern hybridization and PCR. **A.** DNAs from hairy roots of Peking (lanes 1 and 3), nts382 (lane 2), Bragg (lane 4) and uninoculated roots of Peking and nts382 plants (lanes 5 and 6, respectively) were digested with *Hind*III and probed by *rolC* gene of *A. rhizogenes* strain A4. **B.** Total DNA isolated from hairy roots probed by GUS gene. DNAs from three Peking line (lanes 1-2, 3-4 and 5) and two Bragg lines (lanes 6 and 7) were digested with *Eco*RI (lanes 1, 3, 5, and 6) or *Hind*III (lanes 2, 4 and 7). Lane 8 was untransformed Bragg roots restricted with *Hind*III as a control. The signals, located only in high molecular DNA region due to poor digestion in lanes 1 - 4, indicate integration of T-DNA. Lane 6 (Bragg) was negative for the GUS probe. **C.** *rolB* fragment was amplified from the K599 lysate (lane 1) and transgenic Peking root DNA (lane 2), but not from the untransformed control Peking sample (lane 3). M: DNA kb ladder marker.

Conclusions

We were especially interested in the question of whether the high frequency of soybean root transformation with the K599-based system was consistent, because of the well-documented difficulty in soybean transformation and regeneration. A positive answer to the question means a potentially wide application of this system in nodulation research. The abnormal nodules with prolonged meristematic activity could also have significant implications on gene interaction and its role in regulation of organogenesis.

TRANSFORMATION FREQUENCY

High frequency of soybean root transformation by K599 and K599 GUS-I was reproduced in separated experiments. When cultivar Peking was used, 70 % or more of plants with transgenic hairy roots could be easily achieved, proving that this is a good system to study root-related events. Most nodules with normal morphology formed on hairy roots of either cultivar Peking or Bragg were functional as demonstrated by the fact that transgenic nodules reduced nitrogen at a rate similar to that of wild-type nodules. Although supported by transgenic roots, no altered phenotypes in the wild-type shoot of chimeric plants were observed.

Though the high frequency in hairy root induction from Peking was relatively consistent, fluctuations were also observed from experiment to experiment. In some experiments, less than half of the plants produced hairy roots while in others nearly all plants were transformed. Such variation was thought to be caused, in part, by the ambient conditions during inoculation, such as temperature and moisture. The latter was more critical for successful invasion of bacteria. In a couple of trials, the plants wrapped in cotton wool soaked with bacterial suspension around the wound site gave higher percentage of hairy roots. Other factors influencing the transformation frequency include inoculation techniques, namely choice of seedling age, position of the hypocotyl for inoculation (distal or basal to cotyledon attachment site), and types of cells. In soybean hypocotyls and roots, surface or sub-epidermal cells perhaps are more susceptible to *Agrobacterium* infection and differentiation (Finer *et al.*, 1996). The time interval between wounding and bacterial application could also make a difference in transformation frequency.

ABNORMAL NODULES ON TRANSGENIC HAIRY ROOTS

Abnormal nodules were harvested originally (Bond, 1993) only from Peking inoculated with *A. rhizogenes* strain K599 and K599 carrying a binary vector containing a chalcone synthase cassette (K599::CHS). The follow-up study showed that both Peking and Bragg have the ability to give rise to meristematic nodules. This result proved that the mutant nodule phenotype was not cultivar-specific. The abnormal nodules were also observed on cultivar Williams transformed with K599 (Dr. Peter Straube, Washington University, St. Louis, MO, personal communication).

The absence of meristematic nodules in hairy roots, transformed with K599 harboring a GUS-intron plasmid (K599::GUS-I) in an earlier study was thought to be due to the interference of the binary vector with the Ri plasmid T-DNA gene action. The speculation may still hold despite the fact that abnormal nodules expressing β -glucuronidase activity were obtained (Figure 2.1B). The frequency of blue abnormal nodules was low. The possibility that gene interaction occurred between binary vector p35SGUS-I and Ri T-DNA and affected T-DNA integration cannot be excluded. In all experiments, meristematic nodules were produced only in a smaller proportion (less than 10%) of transgenic roots. At this moment there is no clue for any explanations. A speculation is that T-DNA random insertion could exert a positional effect at some sites on nearby genes controlling nodule development.

Acetylene reduction, which is routinely used to estimate the capability of nodules to fix atmospheric nitrogen, showed that abnormal nodules were capable of fixing nitrogen, although at substantially lower rates. Some of such nodules were totally inactive. Premature senescence could be an explanation, as Bond observed that abnormal nodules senesced earlier than wild-type ones. This experimental outcome led to the conclusion that the nitrogen-fixing ability in abnormal nodules was impaired. It is also likely that interference of T-DNA genes (including *rol* genes) with nodulation pathways not only reshapes nodule morphology, but also influences cell differentiation, which may change cells' physiology and metabolism, and ultimately, affect bacteria invasion and N_2 -fixing function.

It is now accepted in general that nodule development is controlled by plant genes. The abnormal nodules that emerged from transgenic hairy roots raise possibility that bacterial genes expressed in plant cells can exert an effect on nodule development. The cause was immediately assumed to be the *rol* genes of *A. rhizogenes* as these genes are well-known to cause alteration in organ development in transgenic plants. Because alteration similar to the meristematic nodules has not been reported in *A. rhizogenes*-mediated root transformation in other plants, it can also be argued that other T-DNA-located genes could have an effect on nodule development. Interestingly, none of the *Lotus japonicus* transgenic (hairy root) lines, including the ones induced by K599, showed such abnormal nodules. However, *L. japonicus* was not efficiently transformed with K599. Apparently the formation of meristematic nodules is (cucumopine-type) K599 dependent. The copy number (dose effect) and positional effect of integrated T-DNA, which could affect expression of either T-DNA genes (*rol* genes) or soybean nodulation genes, might be the cause of the small proportion of abnormal nodules observed on transgenic roots.

The complication of study increases as participating components/factors increase. In this system three species of organisms (soybean, *A. rhizogenes* and *Bradyrhizobium japonicum*) were involved. This made genetic analysis of the cause of abnormal nodule phenotype difficult, let alone, we know very little about bilateral gene interactions. The *rol* genes, and perhaps also other T-DNA genes, are believed to be involved in the induction of abnormal nodules. A transgenic soybean hairy root appears to be a prerequisite, because K599 added externally or mixed with *Bradyrhizobium japonicum* USDA110 did not alter nodule morphology. To understand the mechanism(s) underlying this unusual phenomenon, it is necessary that the identities and functions of individual genes in the Ri T-DNA of K599 be elucidated.

Soybean transformation, in general, was rated as "inefficient but consistent" (Finer *et al.*, 1996). For this particular *A. rhizogenes* transformation system I would rate it "efficient and consistent, but not perfect". This root transformation system is now adopted by many laboratories around the world (D.P. S. Verma, Ohio State University; H. Kouchi, Japan; A. Pühler, Germany; B. J. Carroll, Australia; E. Stroe, Romania).

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Part III

***LOTUS JAPONICUS* ROOT TRANSFORMATION AND SPONTANEOUS
REGENERATION OF TRANSGENIC PLANTS FROM HAIRY ROOTS
INDUCED BY *AGROBACTERIUM RHIZOGENES* STRAIN AR12**

Abstract

Lotus japonicus is model legume producing determinate type nodules. We demonstrated that susceptible to *Agrobacterium rhizogenes*. High frequency transformation was obtained in two weeks from most *A. rhizogenes* strains tested. Transgenic plants emerged spontaneously from the liquid culture of hairy roots induced by strain AR12 but not from root lines induced by closely related strains. It took only about three months from inoculation with *Agrobacterium* to obtain relative large plants (10 cm and more in height). This method is less labor-intensive because no frequent subcultures/transfers is required. Many regenerants can be produced from a 50 - 100 ml hormone-free liquid culture without refilling the medium. PCR and Southern hybridization analysis confirmed that these plants are transgenic.

Introduction

Using a simple system to study complicated biological processes to discover common mechanisms underlying growth and responses of organisms to internal and external stimuli is a standard practice in biological studies. In plant sciences, *Arabidopsis thaliana* as a model for molecular biology research (Koncz *et al.*, 1992) has helped tremendously with our understanding of developmental biology (Celenza *et al.*, 1995, Szekeres *et al.*, 1996, Yanofsky 1995), plant responses to environmental stimuli (Chamovitz and Deng, 1995) and pathogenic plant-microbe interactions (Bent *et al.*, 1994, Mindrinos *et al.*, 1994). However, the inability of *Arabidopsis* to interact symbiotically with nitrogen-fixing bacteria *Rhizobium/Bradyrhizobium* limits its use in nodulation and nitrogen fixation research (Kolchinsky *et al.*, 1994). Unlike interaction between plant and pathogenic microorganisms, mycorrhizal fungi or endophytic bacteria, legume-*Rhizobium/Bradyrhizobium* interactions uniquely result in the formation of a new organ, the nodule. The unique structure and role of nodules reflect an example of advanced plant-bacteria co-evolution. The plant defense mechanism commonly seen in plant-pathogen interactions is suppressed during *Rhizobium* invasion. Nodules protect and nourish the bacteroids in the infected cells so the latter can fix atmospheric nitrogen into plant usable ammonia. Differing from initiation of lateral roots from pericycle cells and new organs like flowers and tendrils from cambial cells, nodule formation in legumes starts in the root

cortical cells. Therefore it is a non-preprogrammed event (Gresshoff, 1993), subject to internal and external modulation, such as phytohormones (Hirsch and Fang 1994), nitrate inhibition (Carroll and Gresshoff, 1983) and autoregulation (Carroll *et al.*, 1985).

Soybean and alfalfa have been studied extensively for nodulation and *Rhizobium*-legume interaction. The physiology and genetics of both legumes *per se* and their symbiotic partners *Bradyrhizobium japonicum* and *Rhizobium meliloti* are well known. Transformation and regeneration of transgenic plants have been demonstrated with both plants (Chee *et al.*, 1995; Steward *et al.*, 1996; Austin *et al.*, 1994; Hill *et al.*, 1991). However, the tetraploidy of alfalfa and the ancestral tetraploid genome of soybean made genetic analysis complicated. A high efficiency protocol for *A. rhizogenes* K599-based root transformation was developed by Bond *et al.* (1998). With special soybean genotypes such as Peking, a transformation frequency of 90% and more, measured by hairy root induction, can be obtained. Nonetheless, it takes four weeks to produce hairy roots in Peking and even longer period from other genotypes. Besides, regeneration of transgenic plants from hairy roots has not succeeded and transgenic lines cannot be maintained and propagated. A legume with simple genome, fast growth rate, and ease for transformation and regeneration will be greatly beneficial to research in this area.

In recent years *Lotus japonicus* (related to the forage legume birdsfoot trefoil) and *Medicago truncatula* (also a forage crop, Australian barrel medic, related to alfalfa) have been adapted as 'model' legumes (Barker *et al.*, 1989; Sagan *et al.*, 1996; Handberg and Stougaard, 1992; Jiang and Gresshoff, 1993; 1997) because of their biological and genetic merits for experimentation. Both are diploid, autogamous and have short life cycle, small genome size (about 0.4-0.5 pg DNA/haploid genome), and are flexible in terms of growth condition required. While *M. truncatula* forms indeterminate nodules (Penmetsa and Cook, 1997), *L. japonicus* gives rise to determinate ones, in which nodule meristematic activity ceases during early stage of nodulation.

We started to use *Lotus japonicus* as model plant to investigate the molecular aspects of nodulation in determinate type legumes in 1992. This plant has all desired features for molecular as well as classical genetic research. Its small size makes it easy to handle in laboratory conditions. The life cycle (from seed to seed) can be completed in about three months. Large flowers and long blooming period are favorable for crossing.

Spherical nodules can be seen one week after *Rhizobium loti* inoculation and harvested in two more weeks. Earlier, we demonstrated efficient sexual hybridization, detection of molecular polymorphism, skeletal map construction, and development of recombinant inbred lines for *L. japonicus* (Jiang and Gresshoff, 1997). High frequency of transformation of this plant was achieved by *Agrobacterium*-mediated T-DNA transfer techniques and success in regeneration of transgenic plants have been reported (Thykjaer *et al.*, 1995). However, the current regeneration procedures are tedious, requiring frequent subculture and several media with different composition of phytohormones and other substances (Handberg *et al.*, 1994; Stiller *et al.*, 1997).

Recently Stiller *et al.* (1997) extended transformation methods from using *A. tumefaciens* to *A. rhizogenes* and improved the regeneration technique in *Lotus* developed by Handberg *et al.* (1994). High frequency transformation with both *Agrobacterium* species has been achieved and regeneration period has been shortened from 8 to 6 months for *A. tumefaciens* transformed hypocotyls. A comparable protocol for regeneration from *A. rhizogenes* induced hairy roots is also available (Stiller *et al.*, 1997). Oger *et al.* (1996) took advantage of the ability for spontaneous regeneration of the *L. japonicus* cotyledon attachment site and developed a new method with which regenerant plantlets were obtained in 9 to 12 weeks. Here we report a fast and even simpler method to produce transgenic *L. japonicus* plants from *Agrobacterium rhizogenes* strain AR12-induced hairy roots in relatively short time without any callus stage, which reduces the chances of somaclonal variation and chromosomal changes.

Materials and Methods

PLANT MATERIALS AND BACTERIAL STRAINS

Agrobacterium strains used in this study and their relevant phenotypes are listed in Table 2.1. Bacteria were grown in modified Bergersen's medium (BMM: Na_2HPO_4 360 $\text{mg}\cdot\text{L}^{-1}$, $\text{MgSO}_4\cdot 7\text{H}_2\text{O}$ 80 $\text{mg}\cdot\text{L}^{-1}$, $\text{FeCl}_3\cdot 6\text{H}_2\text{O}$ 3 $\text{mg}\cdot\text{L}^{-1}$, $\text{CaCl}_2\cdot 2\text{H}_2\text{O}$ 40 $\text{mg}\cdot\text{L}^{-1}$, $\text{MnSO}_4\cdot 4\text{H}_2\text{O}$ 0.1 $\text{mg}\cdot\text{L}^{-1}$, H_3BO_3 0.03 $\text{mg}\cdot\text{L}^{-1}$, $\text{ZnSO}_4\cdot 7\text{H}_2\text{O}$ 0.03 $\text{mg}\cdot\text{L}^{-1}$, $\text{NaMoO}_4\cdot 2\text{H}_2\text{O}$ 0.0025 $\text{mg}\cdot\text{L}^{-1}$, $\text{CuSO}_4\cdot 5\text{H}_2\text{O}$ 0.0025 $\text{mg}\cdot\text{L}^{-1}$, $\text{CoCl}_2\cdot 6\text{H}_2\text{O}$ 0.0025 $\text{mg}\cdot\text{L}^{-1}$, biotin 0.1 $\text{mg}\cdot\text{L}^{-1}$, thiamin 1 $\text{mg}\cdot\text{L}^{-1}$, mannitol 3 $\text{g}\cdot\text{L}^{-1}$, sodium gluconate 0.5 $\text{g}\cdot\text{L}^{-1}$, Yeast extract 0.5 $\text{g}\cdot\text{L}^{-1}$, Bacto Agar 10 $\text{g}\cdot\text{L}^{-1}$, pH 7.0) for 3 - 5 days. Seeds of *Lotus japonicus* (accession number

B-129) 'Gifu' was originally obtained from Dr. Jens Stougaard, Aarhus University, Aarhus, Denmark and since were propagated in a greenhouse in Knoxville, TN. After scarifying with sand paper, they were surface-sterilized in 70% ethanol and 3% hydrogen peroxide for 10 minutes followed by five rinses of sterile water. The surface sterilized seeds were then placed on a stack of wet filter paper in a Petri dish and germinated for three to five days in the dark (20-25 °C). Seedlings (10 - 20 mm long) were transferred to half-strength Gamborg's B5 (Sigma, St. Louis, MO) slope agar plates and grown for 2 more days before inoculation with *Agrobacterium*. All media used in this study were free of plant growth regulators.

TRANSFORMATION AND REGENERATION

Before inoculation, bacterial cells (3 days' culture) were spun down and resuspended in 1 mL fresh BMM. One microliter of bacterial suspension (approximately 10^{10} cfu mL⁻¹) was applied to the hypocotyl region. The bacteria-coated area was then punctured gently with a 26 gauge needle for 2 - 3 times. The inoculated seedlings were grown in a growth chamber with a 16/8 hours light/dark regime at constant 20 °C. After 10 to 14 days the hairy roots were examined with a stereoscope (Olympus model B061). The untransformed tap root was removed from the mosaic plants when hairy roots were 5 mm or longer to promote growth of the latter. The plants with transgenic hairy roots were then transferred on a fresh, full strength B5 plate containing 2% sucrose and 400 µg mL⁻¹ cefoxamine (Claforan, Germini Bio Products, Calabasus, CA), or 500 µg mL⁻¹ carbenicillin for one to two weeks. A hairy root segment about 2 cm long was excised from the plant and propagated on solid plates, or in 50 - 100 mL liquid medium of the same ingredients in a 250 mL Erlenmeyer flask. Selection of transgenic hairy roots was conducted by using medium in the presence of 100 µg mL⁻¹ kanamycin for the AR12-induced lines, or antibiotics in accordance with the resistance gene carried by the T-DNA/binary vector. Proliferation of hairy roots was achieved by incubating the liquid culture on a rotary platform (100 rpm, dark) in room temperature. To prepare duplicates for liquid culture of the same transgenic root line, root segments taken from plants were grown on plates for two weeks before liquid culture.

Regenerant plantlets emerged from liquid culture with cultured root attached were excised and grown on a hormone-free 1/2 B5 plate containing 1% sucrose for 2 - 3 weeks

before transferring to soil consisting of 50% vermiculite (Palmetto Vermiculite Co. Inc., Arcadia, LA), and 50% pro-mix BX (Premier Horticulture Inc., Redhill, PA) in the greenhouse.

PCR, SOUTHERN HYBRIDIZATION AND GUS ASSAY

DNA extraction and purification

DNA extraction method was based on Dellaporta *et al.*, (1983). Half gram of young leaves or 1 gram fresh root tissue was excised with scissors, frozen immediately in liquid nitrogen and stored in -70 °C deep freezer until DNA isolation. The plant materials were grounded to fine powders with liquid nitrogen in the presence of 0.5 gram of polyclar AT (PVP) and transferred to a 40 mL centrifuge tube. Fifteen milliliters DNA extraction buffer (100 mM Tris-Cl pH 8.0, 50 mM EDTA and 500 mM NaCl) and 1 mL 20 % SDS were added and mixed, the tube was then incubated in 65 °C for one hour. After incubation, the extraction mixture was neutralized by adding 5 mL 5 M potassium acetate (KOAc, pH 8.0) and chilled on ice for 30 minutes. The dirt (precipitated out from extraction mixture containing cell debris and SDS-KOAc complexes) was filtered out through a pleated filter paper (Schleicher & Scheull). DNA and soluble macromolecules (mainly polysaccharides) were precipitated with 0.5 volume of cold (-20 °C) isopropanol. After incubation in -20 °C for 30 min., DNA was pelleted at 12,000 rpm for 15 min., 4 °C. The supernatant was decanted and the pellet was allowed to (partially) dry in room temperature for 1 hour and then resuspended in 0.7 mL chromosomal buffer (50 mM Tris-HCl pH 8.0 and 10 mM EDTA). The suspension was centrifuged in a benchtop microcentrifuge at maximum speed for 10 min. to remove polysaccharides. One tenth volume of 3 M NaOAc, pH 7.5 and 0.5 mL isopropanol were added to the solution and mixed gently but thoroughly. DNA was precipitated by spinning in an Eppendorff microcentrifuge at full speed for 5 min. room temperature followed by 2 washes with 70 % ethanol and dried in a Speed-Vacuum Drier. The DNA pellet was dissolved finally in 100 - 200 µL TE.

When DNA was extracted from root tissue containing higher polysaccharides content, a modification (Li *et al.*, 1994) was employed. After the first precipitation, the pellet was dissolved in chromosomal buffer, treated with RNase A (50 µg/mL¹, Amersham, Arlington Heights, IL) in 65 °C for 15 min., passed through a Sephacryl S-

1000 (Sigma) column by spinning at 200 g_n for 2 min., and precipitated by 10 % PEG 8000 and 1 M NaCl.

PCR detection of the marker genes in the transgenic plants

To determine the transgenic status of regenerant plants by polymerase chain reaction (PCR), primers were designed from the internal sequences of *rolB* and *C* genes of agropine type A. *rhizogenes* strain A4 (Furner *et al.* 1986). A total DNA of 100 ng from plants, 5 ng from plasmids, or 2 µL bacterial lysate was used as template for amplification. Primers (*rolB* forward: 5' CTATTCCTTCCACGATTTCAACC 3', reverse: 5' CTCCAGCAAGT-GAATGAACAAGG 3'; *rolC* forward: 5' TCGATGGATATTGACGAAGAAGG 3', reverse: 5' TGCACTCGCCATGCCTCACCAAC 3') were added to a total volume of 50 µL PCR reaction [10X PCR reaction buffer 5 µL, dNTP 50 µM each, primer 50 nM, MgCl₂ 2 mM, Amplitaq DNA polymerase 1 unit (Perkin-Elmer)]. The samples were preheated at 95 °C for 5 minutes followed by 95 °C for 20 sec., 58 °C for 30 sec., 72 °C for 2 min. 15 sec. (35 cycles) and 72 °C for 7 min. A pair of primers (forward: 5' CGTTTTCAAGTTCTCACCTCTGG 3' and reverse: 5' TTTGTTTGCCTGCCTCGTCA-TAG 3') for bacterial *virD4* gene (Hirayama *et al.* 1988) was used as negative control to examine possible contamination of *Agrobacterium* in plant samples with the same reaction condition as mentioned above. The *rol* gene-containing plasmids pPCV002-AC, pPCV002-C and pPCV002-ABC were used as positive control.

Examination of transgene by Southern blot analysis

In the genomic Southern analysis, six micrograms of total DNA was digested with *Bam*HI or *Eco*RI, run on a 0.9% agarose gel, blotted onto the Zeta-nitrocellulose membrane (Bio-Rad, Hercules, CA) and hybridized by the probe according to the manufacturer's instruction.

For probe preparation, amplified or digested *rolC* fragment from plasmid pPCV002-C was labeled with α-³²P using a Megaprime DNA Labeling System kit (Amersham). To 25 - 50 ng DNA in 5 µL TE buffer, 5 µL primer solution was pipetted into it and the DNA was then denatured at 95 °C for 5 min. The DNA-primer solution was chilled on ice followed by adding 5 µL reaction solution, 4 µL of each dCTP, dGTP and dTTP (0.5 mM), 5 µL α-³²P dATP (3,000 mCi mM⁻¹) and 2 units of DNA polymerase (Klenow fragment). Labeling was performed by incubating the reaction solution at 37 °C

for 15 min. Unlabelled nucleotides were removed by passing the reaction solution through a Sephadex G-50 column (Boehringer-Mannheim, Indianapolis, IN). Incorporated radioactivity was measured with a Beckman liquid scintillation counter (model LS-3801). Approximate of 10^5 cpm (counts per minute) per square centimeter membrane was used in Southern blot analyses. Hybridization was performed at 62 °C for 24 hours. Higher stringent wash condition (67 °C) was employed in Southern blotting for PCR amplified products while lower stringency (60 °C) was adapted in genomic Southern to save autoradiographic time.

GUS assay for AR12-induced hairy roots

The GUS assay was performed according to Jefferson (1987). One piece of root about 1 cm long was cut and immersed in GUS assay buffer (50 mM potassium phosphate, pH 7.0; 1 mM EDTA; 1 mM ferrocyanide; 1 mM ferricyanide; 0.1% Triton X-100; 5 mg X-glucuronic acid per 20 mL solution) in a Falcon 24-well plate (Becton Dickinson, New Jersey). The plate was then sealed with tape and incubated in 37 °C overnight. β -glucuronidase activity was determined under a stereoscope for the blue color.

Results

Lotus japonicus B-129 'Gifu' was previously transformed by *A. tumefaciens* (Hansen *et al.*, 1989). The protocol for increased transformation, more efficient root culture procedures and plant regeneration based on root transformation with *A. rhizogenes* is given (Fig. 3.1). 'Gifu' seeds were germinated aseptically on water saturated filter paper for 3 - 5 days, then transferred and grown on agar slope plates for additional 2 days. The hypocotyl regions were stabbed with 26 gauge needle contains inoculum of *Agrobacterium*. All ten bacterial strains used in this study (Table 2.1) induced hairy roots on *L. japonicus*, confirming its susceptibility. Interestingly strain K599 transformed soybean cultivar 'Peking' at a frequency up to 90 % (Bond *et al.*, 1996), but infected 'Gifu' poorly (Table 3.1). Hairy roots developed in seven to ten days from plants infected with strains 15834 (wild type) and LBA9402, while they appeared in 2 to 3 weeks when inoculated with the other strains. The newly emerged hairy roots were easily distinguished from non-transformed roots by their fresh, whitish color and abundance of root hairs (Fig. 3.2A). Tap roots nontransformed were cut off to promote growth of the putative transgenic hairy root creating a chimeric plant.

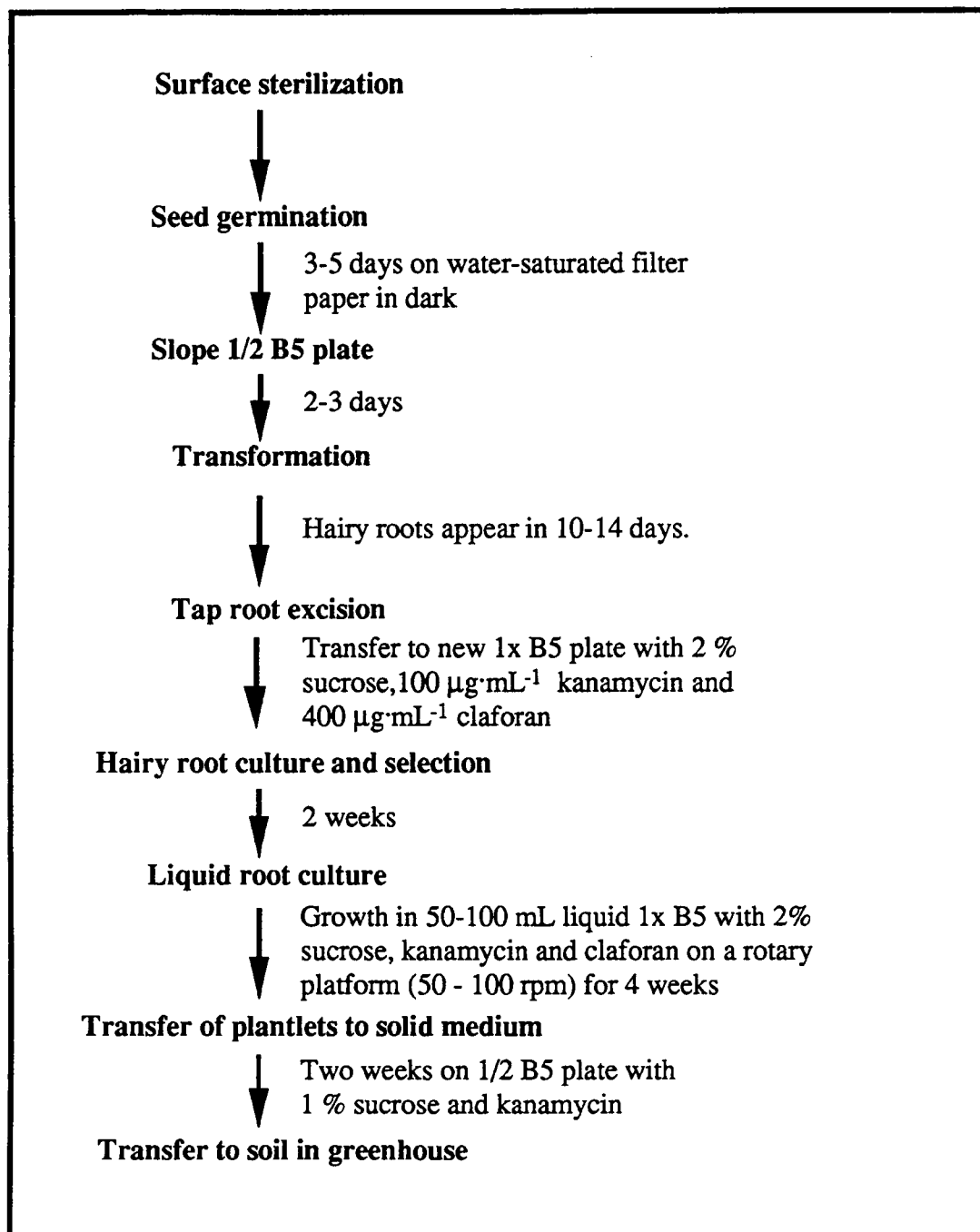


Figure 3.1. A protocol for transformation and regeneration of transgenic *L. japonicus* (based on ecotype 'Gifu') from hairy roots induced by *A. rhizogenes* AR12. The transformation procedure is not different from the one by Stiller *et al.*, (1997) for hairy root induction using other *Agrobacterium* strains. A propagation step on solid medium (B5 plus antibiotics and sucrose) was incorporated to reduce bacterial contamination, but is not necessarily required for liquid culture and regeneration.

The transgenic status of hairy roots was also verified by expression of the β -glucuronidase (GUS), antibiotic resistance (kan^r), and other molecular biological techniques (PCR, as well as Southern blotting for *rolB*, *rolC*, *virD* or GUS genes). Root segments of about two centimeters in length from fast growing hairy roots were used as an inoculant of solid or liquid cultures of hormone-free B5 in the presence of kanamycin 100 $\mu\text{g mL}^{-1}$ (kan) and claforan 400 $\mu\text{g mL}^{-1}$ or carbenicillin 500 $\mu\text{g mL}^{-1}$. On solid medium, a 10 cm diameter Petri dish was covered by root growth in two weeks. In liquid culture, the root mass filled the 100 mL aqueous volume of the container (250 mL Erlenmeyer flask) in 2-3 weeks.

Although hairy roots can be transferred directly to liquid culture containing the antibiotics kanamycin plus claforan or carbenicillin, contaminating *Agrobacterium* often proliferated. In most cases root portion not in contact with the medium in the hairy root promotion plates retained bacteria. Thus root segments were routinely grown first on 1.2% agar-antibiotic plates to restrict or eliminate of contamination. High density of bacteria in root sections exposed to air (therefore void of antibiotics) can be estimated by the quick appearance and high frequency of *A. rhizogenes* colonies formed on antibiotics-free, or even on antibiotics-containing plates. Under such circumstance root growth halts and root tissues deteriorate rapidly. For this reason only root segments in contact with or inside the agar were excised and placed into liquid medium. The bacterial-free status was further checked by growing roots on control plates without antibiotics as well as PCR testing for the absence of the *Agrobacterium virD* gene.

Table 3.1. Infectivity of different *Agrobacterium* strains on *L. japonicus* 'Gifu' and soybean cv. Peking and Bragg.

Strains	hairy root induction strain (% of plants)		
	Gifu ^a	Peking ^b	Bragg ^b
9402	90-100	0	0
15834	70-90	0	0
AR10 (AR12, 14)	50-90	0	0
8196	20	0	0
ARqual	50	< 10	0
A4RSII	80-90	< 10	0
K599	10	50 - 100 ^c	20 - 70 ^c

^a 30 - 50 plants tested per strain. ^b 20 plants tested. ^c based on more than 300 plants tested.

The liquid culture of hairy roots enabled the production of an adequate quantity of transgenic plant materials in short time for various molecular biological studies. Massive fresh root tissue can be harvested in 2 - 3 weeks. Surprisingly, plantlets developed directly from hairy root lines induced by AR12 after 4 weeks' culture without an intervening callus induction stage. These plantlets resembled normal growing seedlings with two thickened cotyledon-like leaves and grew into normal looking plants (Figure 3.2B). This development was in contrast to organogenesis as noted by Stiller *et al.* (1997) on hairy root explants or hypocotyl-derived calli with the same AR12-'Gifu' transformation system. Such plant formation was not observed from any other root lines, including those induced by the sibling strains AR10 and AR14, or the parental strain 15834 of AR12. To be certain that these regenerants did not arise from contaminating hypocotyl tissues during sectioning of hairy roots, the experiment was repeated and only the distal region of root sections on the solid medium culture were taken. Again regenerant plantlets emerged in approximately the same period of culture from the root masses. More plantlets developed from all root culture surface areas during subsequent several weeks without replenishing the medium. Five independently induced AR12 root lines were tested in the repeat experiment; all produced plantlets, demonstrating that the spontaneous regeneration was a stable feature of AR12-induced hairy roots culture but not generated from contaminating hypocotyl tissues. The plantlets (with root sections about 5 cm in length still attached) were excised and transferred to a new 1/2 B5 (Gamborg *et al.* 1986) plate containing 1% (w/v) sucrose and 100 mg mL⁻¹ kanamycin and grown for one or two more weeks before being planted in vermiculite in the greenhouse (Figure 3.2C).

To confirm that these regenerants were transgenic, PCR detection and the Southern blot analysis was performed. Primers were designed from the internally conserved sequence of *rolB* and *rolC* genes. On agarose gels a single band from the regenerant comigrated with expected PCR product amplified from bacterial lysates of 15834, AR10, AR12, AR14, as well as several *rolB*- or *rolC*-containing plasmids (Figure 3.3A and B). The *rolB* primers also synthesized a single fragment DNA from samples of *A. rhizogenes* K599 (a cucumopine type strain) and K599 transformed soybean. No specific PCR product was obtained from either wild-type soybean Bragg and *L. japonicus* 'Gifu'. Much less *rol* gene fragments were amplified from plant samples than plasmid and lysate samples. This may be explained by low target template abundance or competition of other

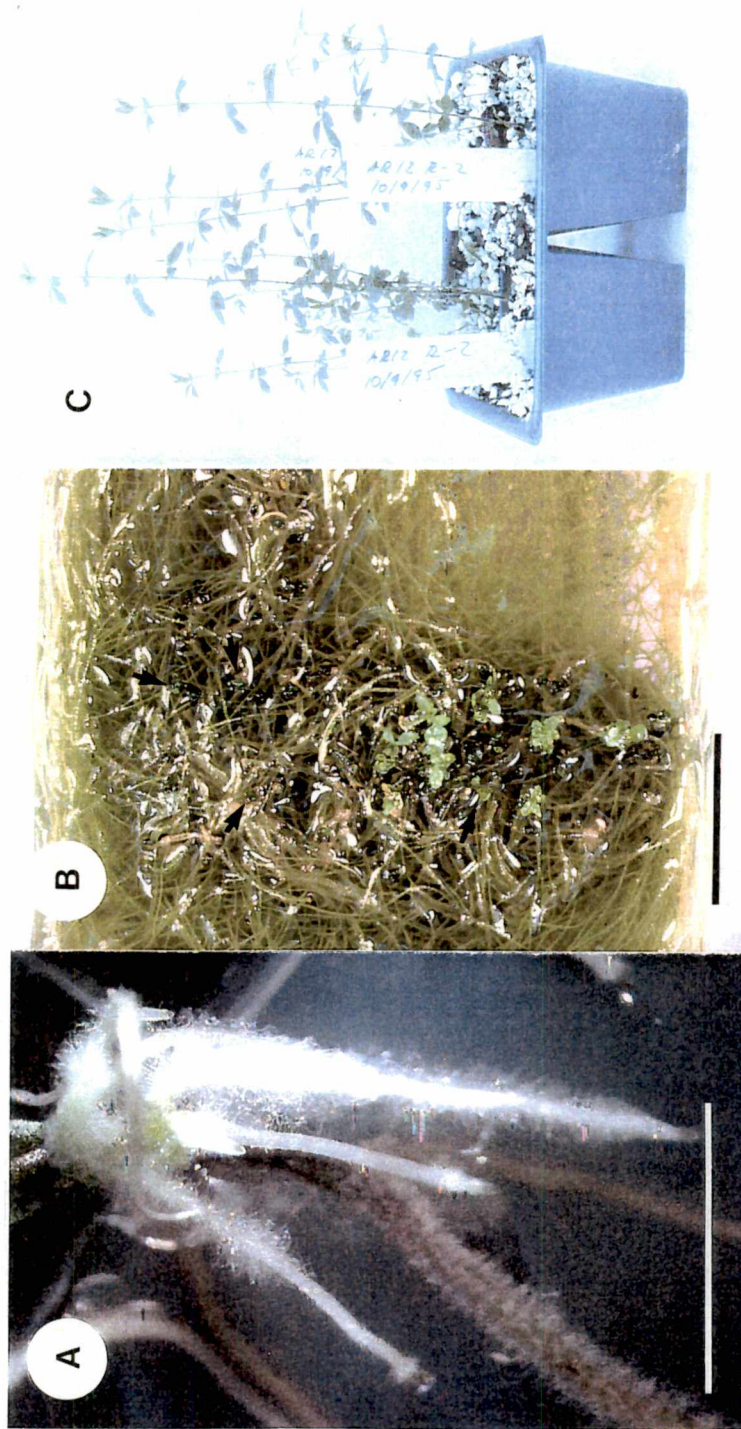


Figure 3.2. Regeneration of 'Gifu' plants from AR12 induced hairy roots. **Panel A**, hairy roots close-up. Hairy roots were easily distinguished by their fresh white appearance from the untransformed primary roots. The callus tissue from which the hairy roots emerged can be seen as greenish in color. The primary roots were cut off and seedlings with hairy roots were grown further on a new plate (1/2 B5 supplied with 2% sucrose and 400 $\mu\text{g.L}^{-1}$ claforan) for one or two weeks. Regeneration of 'Gifu' plants from AR12 induced hairy roots. Tiny plantlets arose from liquid hairy root culture (**panel B**). Arrows indicate cotyledon-like leaves, an implication of embryogenesis. The spontaneous regenerants of about 10 cm tall were transferred to soil in greenhouse (**panel C**). Bar equals one centimeter.

Lotus or soybean sequences for priming sites. Although much weaker in intensity, the band from transgenic 'Gifu' hybridized to the appropriate probe while the band from K599 lysate and K599 transformed soybean failed under high stringency hybridization and wash condition (67 °C, Figure 3.3A, lower panel). The result did not deviate from our expectations and can be explained by our amplification strategy. Higher homologous regions among agropine, mannopine and cucumopine type *rolB* and *C* genes were used for primer design in order to amplify fragments from different Ri plasmids. However, it was not without notice that the amplified regions share relatively low sequence similarity (40 - 60 %) among the genes.

In Southern analysis using genomic DNA as templates, the *rolC* probe hybridized to a band of 1.9 kb from *Bam*HI and 2 bands of 6.2 and 4.8 kb, respectively, from *Eco*RI digests (Figure 3.3C). It is likely that there was a single integration event in the regenerants. The two bands in *Eco*RI digests are likely resulted from a restriction site close to one end of the probe. The existence of a single *Eco*RI site was confirmed by scrutinizing the *rolC* sequence. A faint hybridization signal was also detected from the partially or undigested high molecular weight DNA, indicating genuine integration of the *rolC* gene into the plant genome. Transgenic plant materials from *Agrobacterium*-mediated transformation frequently carry internal bacterial contaminants. To exclude the possibility that the amplified *rol* gene fragments were from contaminating bacteria, PCR using primers for the *A. rhizogenes* A4 *virD4* gene (Slightom *et al.*, 1986) with the same set of samples was also performed. The *virD4* fragment was amplified from all bacterial DNA but not from any plant and *rol* gene-containing plasmid samples (Figure 3.3D). We therefore assure that transgenic plant material was free of agrobacteria.

Legend of Figure

Figure 3.3. Transgenic status of spontaneous regenerant 'Gifu' plants verified by PCR and Southern hybridization. A *rolB* (A, upper panel) and *rolC* (B) fragment was amplified from bacterial lysates, *rol* gene-containing plasmids pPCV002-ABC, pPCV002-B1000 and regenerant 'Gifu' plants. Samples from wild-type *Lotus* and soybean, in contrast, failed to amplify DNA from *rolC* primers or produced non-specific PCR products, and no distinct band could be recognized. Although a PCR product was detected from the cell lysate of cucumopine type strain K599 using primers derived from the agropine type (strain A4) *rolB* gene, it failed to hybridize to the A4 *rolB* probe under high stringent wash condition (67 °C, A lower panel). Similarly, the hybridization signal was evident only in samples where DNA template was from the transgenic *Lotus japonicus* plants in the genomic Southern blot experiment probed with an agropine type *rolC* fragment (C). The contour feature of the gel documentation system (model IS-1000, Alpha Innotech Corporation, San Leandro, CA) was used to enhance the signal. A single copy of insertion in the transgenic *L. japonicus* plant was demonstrated by the a single hybridization band of about 1.9 kb in the *Bam*HI digest. An *Eco*RI site inside the *rolC* gene gave rise to two bands (4.8 kb and 6.2 kb respectively) with signals whose intensity were proportional to the length of sequences in the probe. PCR of the *virD4* gene (D) was conducted as control and showed that the signal detected by *rolC* was not due to contaminating bacteria.

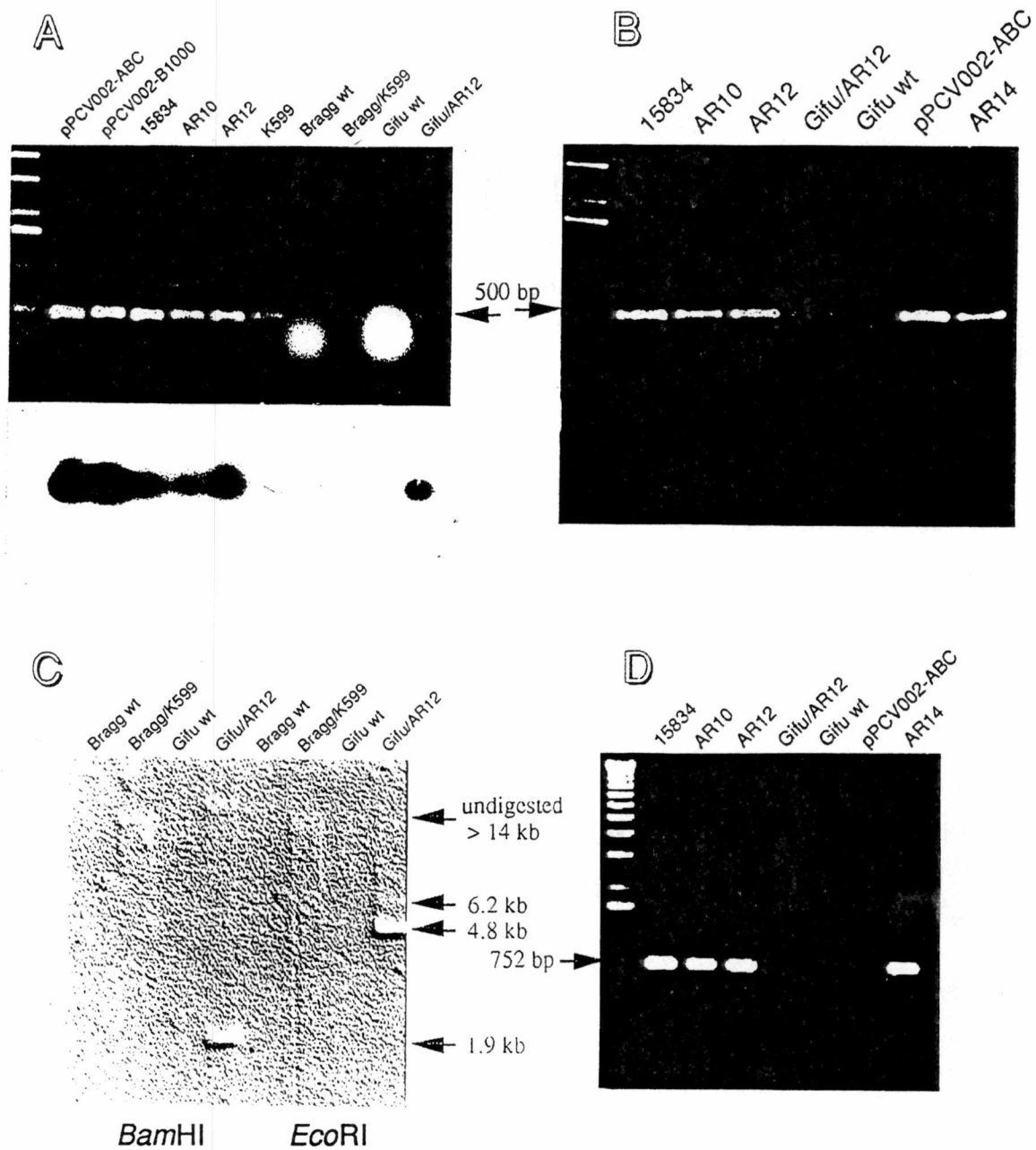


Figure 3.3

Discussion

L. japonicus 'Gifu' was originally transformed by *A. tumefaciens* (Handberg *et al.*, 1994). Here we showed that it is also susceptible to *A. rhizogenes*. Transformation using strains of *A. rhizogenes* and modified *A. tumefaciens* was conducted on soybean and *L. japonicus* 'Gifu'. We initiated *A. rhizogenes*-mediated root transformation of *Lotus* to bypass the difficulties encountered in soybean research, such as lower transformation frequency, cultivar-dependent transformation and regeneration and long regeneration period (Bond, 1993; McDonnell, 1997). Although both are tropical legumes producing determinate type nodules, the differences between two species are obvious when molecular genetic study of nodulation is concerned. Table 3.2 gave brief comparisons between the two plants. As a model legume, *L. japonicus* 'Gifu' received rapid acceptance world-wide since its proposal by Handberg and Stougaard 5 years ago. Important progress was also made in the genetics of the symbiotic partner of *R. loti*, which had been thought earlier to be the weakness of using this system compared with that of *M. truncatula* (nodulated by *R. meliloti*) and soybean (nodulated by *Bradyrhizobium japonicum*). An about 400 kb chromosomally located symbiotic island of *R. loti* has recently been sequenced (Sullivan and Ronson, 1997). This is undoubtedly giving research in this model system a forceful push. Furthermore the structure of *R. loti* lipo-oligosaccharide Nod factor has recently been revealed (H. Spaink, personal communication), being similar to that seen in *Bradyrhizobium japonicum* (Stacey, 1995; Sanjuan *et al.*, 1992)

We were also motivated by observations of abnormal nodules from *A. rhizogenes* K599 and its derivative K599 GUS-Int-mediated soybean root transformation system and hoped that the *L. japonicus* system would have enabled us to discover the mechanisms controlling the development of the root transformation-associated meristematic nodules in determinate type legumes. In our work with this plant no such abnormal nodules have been detected in any of 'Gifu'-*A. rhizogenes* transgenic lines. This result supports our speculation that meristematic nodules in soybean are *A. rhizogenes* strain-specific.

Interestingly, only the AR12 root lines regenerated spontaneous plants. Despite prolonged culture period, no hairy root lines induced by other strains, including the AR10, AR14 and the parental strain of AR series, 15834, produced regenerants. The AR12 shares

Table 3.2. Features of *L. japonicus* 'Gifu' and soybean 'Peking' related to nodulation study.

	Gifu	Peking
Transformation frequency % (low - high)	80 (50 - 100)	70 (40 - 90)
Hairy root emergence DAI (earliest)	14 (7)	28 (14)
Delay of nodulation (Nodule appearance DAI)	No* (7 - 10)	Yes (30 - 60)
Occasional-meristematic nodules	No	Yes
Genome	diploid	partial tetraploid
Genome size (Mega bp/haploid genome)	350 - 400	900 - 1000

*Estimation based on experience and comparison of seedlings.

Other feature of *L. japonicus* 'Gifu' include:

Short life cycle. Seeds to seeds 3 months

Perennial

Autogamous

Continuing flowering and seed setting

Large flowers, easy to cross.

Small seeded, many seeds per cross.

basically the same genetic background as 15834, AR10 and AR14. The only known difference among these strains is the presence or absence and type of a reporter gene integrated into the Ri-plasmid (Hansen *et al.*, 1989). Instead of the chloramphenicol acetyl transferase (CAT) gene in AR14, a *uidA* (GUS, or β -glucuronidase) gene is used in the AR12. The 15834 is wild type and the AR10 is an auxotrophic mutant (His⁻) of 15834 and carries no reporter gene. The fact that spontaneous transgenic plants arose only from the AR12 induced root lines in two separate experiments demonstrated clearly that the regeneration capability was specific and relied on AR12. However, comparison between the AR12 and closely related strains gave no clue about the causes. It was speculated that the integration of the *uidA* gene into the Ri-plasmid may have exerted a positional effect on neighboring genes or have caused a DNA rearrangement that affected the function of genes controlling plant morphology. Remarkable differences in the growth characters of hairy roots induced by 18534 and the AR series of *Agrobacterium rhizogenes* strains were observed (data not shown). Induction of hairy roots by the wild-type strain 15834 was

much faster and more efficient than by other strains. Infection by both 15834 and AR10 induced bushy hairy roots at the inoculation site. In contrast, only 3-4 transgenic roots emerged from AR12 and AR14 inoculated plants. Hairy roots commonly grow more vigorously than wild-type roots in hormone-free root culture. Our observation suggested hairy root induction is a concerted action of T-DNA genes. Interruption of individual gene or gene function due to positional effect resulted from insertion of foreign gene could cause altered growth characters in transformed tissues.

We described here a method by which abundant regeneration of transgenic *L. japonicus* plants were obtained from hairy root lines transformed with *A. rhizogenes* strain AR12. Compared to most protocols for the regeneration of transgenic plants, it was simpler and faster. The whole procedure from infection with *A. rhizogenes* AR12 to transfer of regenerant to the greenhouse can take as short as three months. No exogenous phytohormone was used and subculturing is greatly reduced. It also avoided the need for tissue culture facilities for callus induction, shoot induction, and root induction. Void of the callus phase was desirable as it may lower the chance of somaclonal variation.

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Part IV

**ISOLATION AND PRELIMINARY CHARACTERIZATION OF A
GENOMIC *ENOD40* CLONE FROM *LOTUS JAPONICUS*
ECOTYPE 'GIFU'**

Abstract

The early nodulin gene *enod40* is a novel plant gene possibly involved in regulation of endogenous hormone balance and induction of cell division during nodule initiation. We reported here the isolation of an *enod40* clone (*Ljenod40*) from model legume *Lotus japonicus* ecotype 'Gifu'. The *Ljenod40* contains the two highly conserved regions identified in all *enod40* clones. A short peptide of 12 amino acids was deduced from the putative ORF in region I. Sequence analysis showed that the promoter of *Ljenod40* shares high homology (about 60 % spanning a region of 500 nucleotides) with that of soybean (*Gmenod40*), including an identical stretch containing the two putative TATA and CAAT elements. A division of two groups of *enod40* gene was proposed based on multiple nucleotide and deduced oligopeptide sequence alignments. Isolation of *Ljenod40*, together with our early demonstration of high transformation and regeneration in *L. japonicus* will enable us to study regulation of hormone signaling and gene expression during nodule induction using a homologous model system.

Introduction

Upon inoculation of symbiotic bacteria *Rhizobium/Bradyrhizobium* or treatment with lipo-chitinoligosaccharide Nod factors, dramatic physiological and cytological changes occur in a zone of legume roots called the nodulation window (Long, 1989; Caetano-Anollés and Gresshoff, 1991; van Rhijn and Vanderleyden, 1995; Spaink, 1992; Denarie and Cullimore, 1993; Rolfe, 1988). Differentiated cortical cells in the infection window resume cell cycle and form cell division loci, which develop into nodule primordia (Charon *et al.*, 1997; Heidstra *et al.*, 1997; Tsien *et al.*, 1983). Concomitant with these changes is the induction of expression of some early nodulin genes (Minami *et al.*, 1996a; Kondorosi *et al.*, 1991). One of the early nodulin genes, *enod40*, has recently caught researchers' attention because of its unique property.

Plant hormones have long been suggested to be involved in nodule development. However, search for the hormone link between *Rhizobium* infection/Nod factor signaling and nodule formation has proven to be much difficult. The *enod40* gene seems to come to fill the gap. *enod40* is expressed in root pericycle and dividing cortical cells in the very

early stage of nodule organogenesis (Kouchi and Hata, 1993; Yang *et al.*, 1993; Crespi *et al.*, 1994), and its transcript begins to accumulate as early as 6 hours after *Rhizobium/Bradyrhizobium* inoculation (van de Sande *et al.*, 1996) or Nod factor treatment (Minami *et al.*, 1996b), before first round cell divisions can be detected. The gene has been isolated from several legume plants including soybean, alfalfa, pea, kidney bean and vetch (Yang *et al.*, 1993; Kouchi and Hata, 1993; Matvienko *et al.*, 1994; Vijn *et al.*, 1995; Papadopoulos *et al.*, 1996), as well as a non-legume tobacco (van de Sande *et al.*, 1996). In most plants it encodes a transcript of 600-700 nucleotides with an open reading frame (ORF) corresponding to a peptide of 12 or 13 amino acids in legume plants, and 10 residues in tobacco. The spatial and temporal expression pattern of *enod40* gene immediately led to the speculation that this gene may play a role in hormone responses. Transgenic alfalfa overexpressing *enod40* induced cortical cell division while transformants overexpressing antisense transcript arrested callus growth (Yang *et al.*, 1997). The function of *enod40* was suggested to equate that of cytokinin (Crespi *et al.*, 1997). In transgenic tobacco protoplast culture, synthetic ENOD40 oligopeptide at subpicomolar (10^{-12} - 10^{-15} M) concentration conferred cells auxin tolerance and was proposed as a novel plant hormone (Crespi *et al.*, 1997; Brewin and Legocki, 1996).

Many legume plants such as soybean, alfalfa, bean, pea and clovers have been subjected to extensive symbiosis and nodulation studies at the molecular level. However, investigations are sometimes difficult to carry out due to various obstacles, such as inefficient transformation and plant regeneration, difficult sexual crossing, a polyploid or large genome, a long life cycle, etc. An *Arabidopsis* equivalent for symbiotic nitrogen fixation and nodulation is clearly of value.

Lotus japonicus, an autogamous, perennial legume appears to have all the merits to fit the requirement: small, diploid genome; short life cycle; flexible for growth conditions. This plant has been used in our laboratory for molecular genetic studies of nodulation as soon as it was proposed as a model legume (Handberg and Stougaard 1992; Jiang and Gresshoff, 1993). Crossing between ecotypes 'Gifu' and 'Funakura' has been demonstrated (Jiang and Gresshoff, 1993). Molecular markers are being identified and genome mapping is in progress (Jiang and Gresshoff, 1997). High efficiency transformation and regeneration protocols are available (Stiller *et al.*, 1997; Chian *et al.*, 1998) and several potential T-DNA tagged nodulation and root development mutants are

under preliminary characterization. Adding to these advances, we isolated a genomic *L. japonicus enod40* gene (*Ljenod40*) aiming to promote *L. japonicus* research and our understanding of nodule developmental biology (Genbank accession number AF013594. Chian and Gresshoff, 1997).

Materials and Methods

ENOD40 GENE PROBE FOR LIBRARY SCREENING

Primer design

Eight *enod40* nucleotide sequences (including two genomic and six cDNA) were obtained from the Genbank database. Multiple sequence algorithm was performed using the PILEUP program of the Genetic Computing Group software package (GCG, The University of Wisconsin). The forward (5'- GTTGGCAAAAATCAATCCATGGTTC -3') and reverse (5'- CCATTGCCTTTTTGTGACTTGCCGG -3') primers based on two highly conserved regions, the Region I and Region II (Figure 4.8) were derived from an alfalfa cDNA clone

PCR amplification of a L. japonicus enod40 fragment

To amplify the *enod40* fragment, fifty nanograms of *L. japonicus* 'Gifu' total DNA was added to a PCR reaction solution containing 50 μ M each dNTP, 100 nM each primer, 2 mM $MgCl_2$, 1X PCR buffer (Perkin-Elmer Cetus), and 1 unit of Amplitaq DNA polymerase in a total 50 μ L volume. Template DNA was denatured at 95 °C for 5 minutes followed by 35 cycles in 95 °C /20 sec., 60 °C or 61 °C/30 sec. and 72 °C/2 min. 15 sec. After the last cycle, the synthesis of the DNA fragment was further extended for an additional 5 minutes in a PTC-200 thermocycler (MJ Research, Inc., Watertown, MA). The amplification product(s) was visualized in 0.8% agarose gel stained with 5 μ g mL⁻¹ ethidium bromide.

Cloning of the PCR product into a plasmid vector

Manufacturers' standard protocols were followed wherever commercial products were utilized. Plasmid pBC SK (chloramphenicol resistant, Stratagene) was digested with *Sma*I (New England BioLabs). The linearized, blunt-ended vector was stabilized by dephosphorylation with heat-sensitive phosphatase HKP (Epicentre Technology). The PCR product was treated as following, before the ligation reaction:

Nucleotide removal, Qiaquick Gel Extraction kit, (Qiagen);

Desalting, Ultrafree[®]-MC filter unit (Millipore);

PCR product polishing, T₄ DNA polymerase (New England BioLabs);

Enzyme/protein removal, Ultrafree[®]-Probind filter unit (Millipore);

The ligation reaction [containing 5 ng pretreated PCR fragment (about 300 bp), 10 ng blunt-ended vector (3.4 kb), 1 μ L T4 DNA ligase (400 units)] was carried in a 12 °C circulating waterbath for 16 hours. The enzyme was then heat-inactivated in 75 °C for 5 minutes.

Competent cell preparation and transformation

Competent cells were prepared according to Sambrook *et al* (1989). Five mL overnight grown *E. coli* strain XL1-Blue MRF'(kan) cells were inoculated into a fresh 100 mL SOB medium containing 20 mM MgSO₄ and incubated at 37 °C with vigorous shaking (300 rpm) for 3.5 hours. Bacteria were pelleted by centrifugation at 4,000 rpm, 4 °C for 10 min. The bacterial pellet was resuspended in 20 mL cold TFB or FSB. After incubation on ice for 10 min., cells were recovered by second centrifugation at 4,000 rpm, 4 °C for 10 min. The supernatant was decanted and cells resuspended in 4 mL medium (TFB or FSB) for immediate or later transformation, respectively.

For bacterial transformation, an aliquot of 100 μ L competent cell suspension was transferred to a prechilled Falcon 2059 tube, mixed with 1.4 μ L freshly made 1:10 dilution of β -mercaptoethanol (Sigma, St. Louis), placed on ice with gentle tapping in every 2 min. After 10 min. incubation, two microliters ligation solution was inoculated into the competent cells and mixed. The bacterial solution was placed on ice for another 30 min. DNA uptake was facilitated by heat shock at 42 °C for 2 min. in a waterbath. After chilling down on ice for 2 min. 900 μ L of SOB with 10 mM MgCl₂ and 10 mM MgSO₄ were added to the Falcon 2059 tube. The transformation reaction was incubated at 37 °C with vigorous agitation (300 rpm) for 1 hour. One- to two-hundred microliters of the transformation solution was streaked on a 90 mm NZCYM-agar plate and grown overnight at 37 °C. Transformants were selected for resistance to 100 μ g/mL⁻¹ chloramphenicol.

PHAGE LAMBDA LIBRARY SCREENING

Preparation of plating culture

The customized phage lambda genomic library FixII (Stratagene, CA) from *Lotus japonicus* ecotype Gifu B-129-S9 was kindly provided by Dr. Jens Stougaard, Aarhus University, Denmark. The average size of *Lotus* DNA inserts was expected to be 12 kb in the *Xho*I site.

To prepare the phage plates, 300 mL NZCYM-agar was poured into a 23 x 23 cm² square Nunc plate (Denmark, distributed by Cole-Palmer, Chicago, IL). The medium was allowed to solidify and then surface-dried for 30 min. in the luminary flow box. The original phage library (titer 2.5×10^7 pfu mL⁻¹) was diluted 100 times. Eighty microliters of the 1:100 dilution were added to 420 μ L SM and mixed with 200 μ L overnight grown *E. coli* XL1-Blue MRA (P) cells plus 300 μ L sterile 0.1 M MgSO₄. The mixture was incubated at 37 °C for 20 min. in a 40 mL Pyrex glass culture/centrifuge tube (Corning). Twenty-five mL top agar (0.7% agarose kept at 47 °C) was pipetted into the tube after incubation period, gently mixed to avoid air bubbles, and immediately poured evenly onto the NZCYM plates. Clear plaques of 0.5 - 1.0 mm diameter appeared on the bacterial lawn after 12 - 16 hours incubation at 37 °C. The plates were cooled to 4 °C for 4 - 24 hours before transferring phage plaques onto membranes. The number of plaques per square plate was estimated to be approximately 100,000, by counting and averaging 5 randomly picked 1 cm² areas. About 400,000 plaques were screened.

Phage DNA transfer

Transfer of phage DNA onto membranes was conducted under sterile condition. A 20 x 20 cm² nitrocellulose membrane (Schleicher & Schuell) was marked asymmetrically with a #2 pencil and laid on the bacterial lawn with phage plaques for 30 seconds. To align the membrane with the Nunc plate accurately, 3 - 4 holes were made by puncturing through the markers at the corners and along the sides into the agar with a 22 gauge needle. Spots corresponding to the holes on the bottom of the Nunc plate were labeled to assist later pinpointing the positive hybridization signals to putative plaques. To eliminate erroneous signals, a duplicate membrane for each plate was used. Transfer of phage plaques from plate to the second membrane was extended to 90 seconds. To transfer phage DNA, the membrane was lifted, plaque-attached side facing up, placed in sequence on solution-

saturated 3M filter papers, for (1) denaturing (Denaturing solution: 1.5 M NaCl, 0.5 M NaOH) 2 min., (2) neutralizing (Neutralizing solution: 1.5 M NaCl, 0.2 M Tris-HCl, pH 8.0) 5 min., and (3) washing (2X SSC: 0.2 M Tris-Cl, pH 7.0) 2 min. respectively. After drying the membranes in room temperature for 30 min., DNA was immobilized in an 80 °C oven for 2 hours under vacuum.

SOUTHERN HYBRIDIZATION AND AUTORADIOGRAPHY

Probe labeling

The 300 bp fragment amplified from *L. japonicus* genomic DNA was used as probe for phage library screening. The probe was radioactively labeled with α -³²P deoxyadenosine triphosphate (α -³²P dATP, specific activity = 3,000 Ci mM⁻¹) using a Megaprime DNA Labeling kit (Amersham). Unincorporated dNTPs were removed with a Sephadex G-50 column (cut off limit 72 nucleotides). The activity of labeled primer was measured with a Beckman Liquid Scintillation Counter (model LS3801).

Hybridization and autoradiography

Membranes with immobilized phage DNA were prehybridized in prehybridization solution (50% deionized formamide, 5X Denhardt solution, 5X SSPE, 0.5 % SDS) for 30 min. The prehybridization solution was then replaced by 100 mL fresh hybridization solution of the same ingredients, plus 100 μ g.mL⁻¹ herring sperm DNA as block DNA. The labeled probe was denatured in 95 °C for 10 min., chilled on ice and added to the hybridization solution at concentration 10⁵ cpm per square centimeter of membrane. Hybridization was performed at 42 °C for 24 hours with gentle agitation. After washing twice with each of wash I (2X SSPE, 1% SDS) and wash II (1X SSPE, 0.1% SDS) for 15 min. at 60 °C, the membranes were slightly dried and sealed in new pouches. Two pieces of Cronex® X-ray film (Du Pont) sandwiching the hybridized membrane were exposed in dark for 2 days. Films with positive signals were aligned to corresponding plates. Agar about 0.5 cm in diameter containing the putative phage clones was picked up with a Pasteur pipette and immersed in 0.5 mL SM with one drop of chloroform in 4 °C for 4 - 8 hours. The titer of the phage suspension was determined by plating a series dilution cultures.

Secondary and tertiary screening

Second and third round (if necessary) screening was performed on 90 mm Petri dishes. The volume of inoculum, medium (bottom agar) and top agar were accordingly reduced. When a positive signal was obtained from a plate with 50 or less plaques, contamination phages from neighboring plaques can be avoid and the tertiary screening usually was omitted. If a plaque was picked from plates containing more than 100 colonies, a third screen was conducted to assure pure phage clones.

PHAGE DNA ISOLATION

Phage DNA was purified with the Lambda Midi Kit (Qiagen, Inc., Chatsworth, CA). About 5×10^5 pfu of purified phage clones in 100 μL XL1-Blue MRA (P) bacterial cells (10^9 cfu $\cdot\text{mL}^{-1}$) was incubated in 37 °C for 20 min., then inoculated into 100 mL NZCYM in a 500 mL flask. The bacteria-phage culture became clear and clumps of cell debris formed after 8 - 12 hours growth at 37 °C with vigorous agitation (250 - 300 rpm). One mL chloroform was added to the culture which was further shaken for 15 min. at 37 °C. Bacterial cell debris and unlysed cells were removed by centrifugation at 8,000 g_n . Degradation of bacterial DNA and RNA was done by adding DNase I and RNase (Boehringer-Mannheim) to the supernatant at a final concentration of 20 $\mu\text{g}\cdot\text{mL}^{-1}$ and 50 $\mu\text{g}\cdot\text{mL}^{-1}$ respectively. Phage particles were precipitated with 10% polyethylene glycol 8000 (PEG 8000) and 1M NaCl in 0 °C for 1 hour followed by centrifugation at 12,000 g_n at 4 °C for 15 min. The pellet was resuspended in 4 mL TM. The phage coat protein was denatured and digested by proteinase K (Boehringer-Mannheim) in proteinase K buffer (50 $\mu\text{g}\cdot\text{mL}^{-1}$ enzyme in 0.01 M Tris, pH 7.8; 0.5% SDS and 5 mM EDTA) at 65 °C for 20 min. The solution was then extracted twice with liquefied and TE buffer saturated phenol (pH 8.0) and twice with chloroform-isoamyl alcohol (chloroform: isoamyl alcohol = 24:1). Purified phage DNA was precipitated with 2.5 volumes of pure ethanol followed by 2 washes with 70 % ethanol and dried in a Speed Vacuum Drier (Savant, SVC-100H).

SUBCLONING OF PHAGE DNA-DERIVED GENE FRAGMENTS INTO PLASMID VECTOR

The purified phage DNA was restricted with *EcoRI* and run in a 0.8 % agarose gel. The fragment containing the *enod40* gene was identified with the amplified probe and the band was sliced from the gel. DNA was isolated using the Gene Clean kit (Bio 101, CA)

and ligated to pBC SK vector predigested with *EcoRI* and dephosphorylated with HKP. Bacterial strain and procedures for transformation of ligation construct were the same as described earlier for PCR product cloning (paragraph 3). Plated bacterial colonies were screened separately for the correct insert by both the amplified and the EST (Szczyglowski *et al.*, 1997) probes, which represent the 5' and 3' halves of the *enod40* transcript, respectively, and are overlapping in the second highly conserved region (Region II, Figure 4.5 and 4.8).

DNA SEQUENCING AND SEQUENCE COMPARISONS

Plasmid DNA was isolated with the QiaQuick Plasmid Spin Kit (Qiagen). The nucleotide sequence of isolated plasmid clones was determined on either strands, both manually with the SequiThermTM Cycle Sequencing Kit (Epicentre Technologies, Madison, WI) and automatically using an ABI MatrixFile automated sequencing device. Search of homologues and comparison of sequences were done via the NCBI Blast service and the FASTA or Comparison software of the GCG package.

Results

PCR AMPLIFIED *ENOD40* PROBE.

The primers used to amplify genomic fragment from *L. japonicus* were based on the cDNA sequence of alfalfa *enod40* (*Msenod40*). Two highly conserved regions in the 5' and middle of the transcripts of the *enod40* (Figure 4.1), respectively, were identified from multiple-sequence alignment algorithms using the PILEUP software of the GCG. These 2 regions were adapted for designing the forward and reverse primers. They are, however, not 100 % homologous among *enod40* clones from different species. Alternative bases were found in several sites. It was justified that a single or two base-pair mismatches would be acceptable and tolerated in PCR reactions. During earlier experiments at an annealing temperature of 60 °C, the control soybean primers gave a single sharp band from soybean total DNA prepared from leaves, while the *Msenod40* derived primers generated a diffuse zone with a core band from *L. japonicus* template (Figure 4.2 A and B). This result was interpreted as one, or possibly two mismatches occurred between primer and template DNA, which caused non-specific priming and produced the diffused zone. Specific priming could be obtained by adjusting the amplification conditions such as temperature, salt and/or primer concentrations. By increasing 1 °C in annealing temperature from 60 to 61 °C, a sharp, single band was amplified from the *Msenod40* derived primers (Figure 4.2C). The identity of the improved PCR product (the sharp band) was confirmed by DNA sequencing. Indeed when the primer sequences were compared against the sequence of *L. japonicus enod40* gene, two base-mismatches was found in the forward primer.

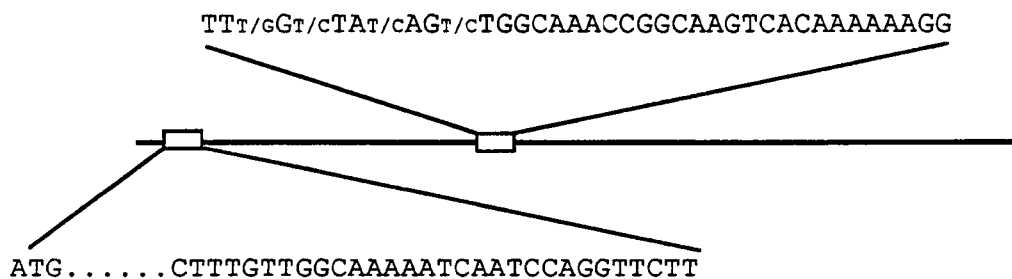


Figure 4.1. Two conserved regions (Region I and Region II) found in all *enod40* genes. The two regions are separated by about 250 basepairs (bp). Alternative bases are indicated by small caps. Non-homologous sequence is shown by dots.

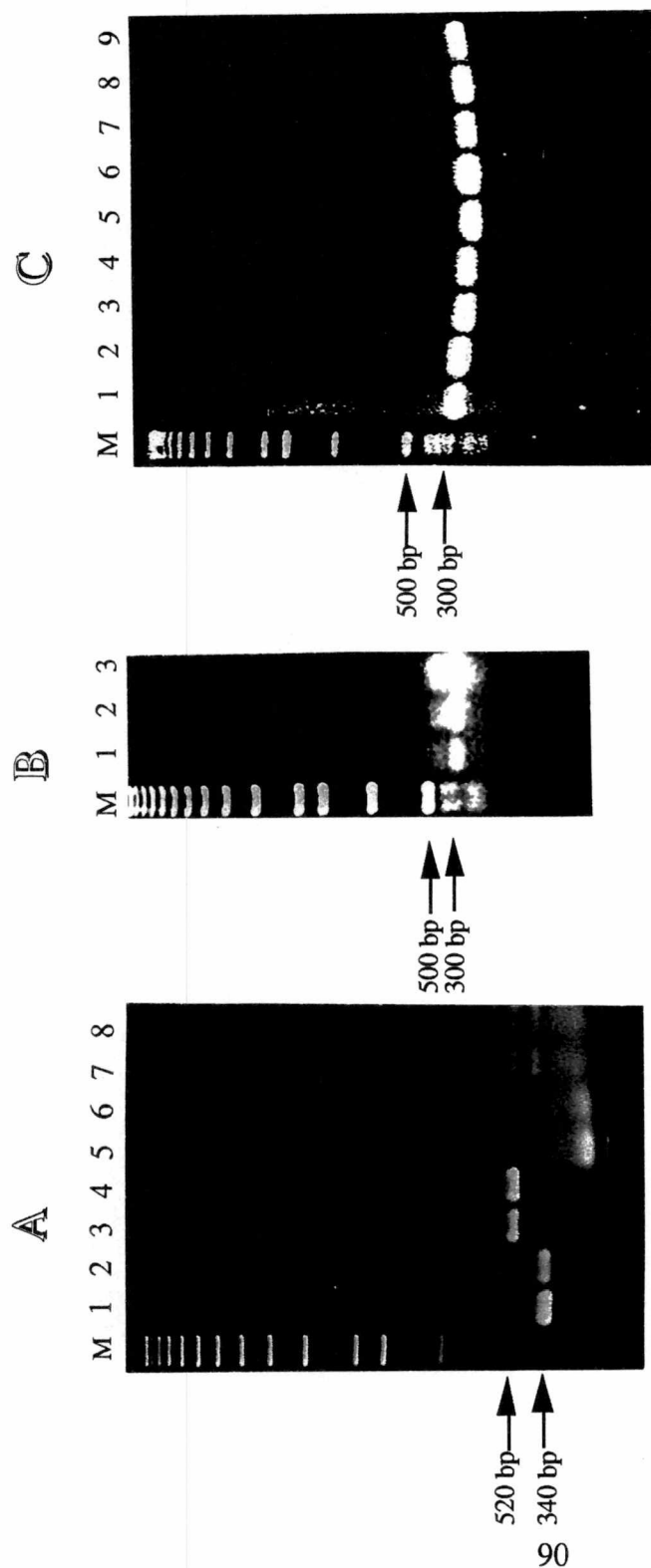


Figure 4.2. PCR amplification of *enod40* fragments. A, PCR products from soybean (lanes 1-4) and *L. japonicus* (lanes 5-8) DNA templates were obtained with soybean primers. The same forward primer F1 was used in all samples. Two reverse primer R1 (lanes 1, 2, 5, 6) and R2 (lanes 3, 4, 7, 8) gave 2 fragments of 340 bp and 520 bp, respectively, from soybean samples. Non-specific amplification in *Lotus* samples was evidenced by multiple bands on agarose gel. B, PCR products of *Ljenod40* using a pair of *Msenod40* based primer. The core band indicated that the primers worked for *Lotus*, while the diffused zone suggested non-specific priming due to 2-base mismatches in the forward primer with template. The reaction was performed at annealing temperature 60 °C. The quantity of template DNA in lane 1, 2 and 3 were 20, 50 and 100 ng, respectively. C, Amplification specificity was improved by increase in 1 °C in annealing temperature to 61 °C using the same primers in B. The DNA templates were: total DNA from *L. japonicus* leaves (lane 1) and *Lotus* 'Gifu' phage 1 genomic clones (lanes 2-9).

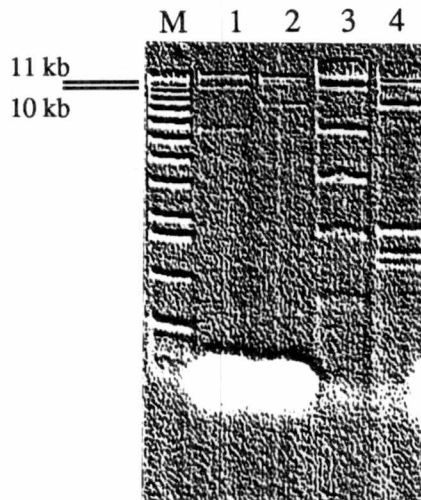


Figure 4.3. Phage DNA restriction. Two DNA preparations of the same clone were digested with *Eco*RI (lanes 1 and 3) or *Kpn*I (lanes 2 and 4). Southern hybridization with PCR derived probe showed the 10 kb *Eco*RI and 11 kb *Kpn*I fragments contained the *enod40* sequence. The bright spots on the bottom of the gel were undigested RNAs.

From approximately 400,000 plaques, four positive signals were detected. However, in subsequent screenings, positive signals appeared only in progenies of 2 clones. Two pure phage clones were isolated after the tertiary screen. They were identical by restriction pattern.

The purified phage DNA was digested with restriction enzymes. Two major bands in the range of 10 - 12 kb from *Eco*RI digest were separated on 0.8 % agarose gels (Figure 4.3). Southern hybridization revealed that the smaller band contained the *enod40* sequences. This fragment was then purified from a gel slice and subcloned into pBC SK plasmid vector linearized with *Eco*RI. From about 2,000 transformed bacterial colonies, two (clones 38 and 57) showed positive

hybridization when probed with PCR generated probe. The same colonies were further confirmed by the EST probe (Szczyglowski *et al*, 1997) which corresponds to 382 nucleotides of the 3' half of the transcript starting from the region II (Figure 4.4, and 4.5).

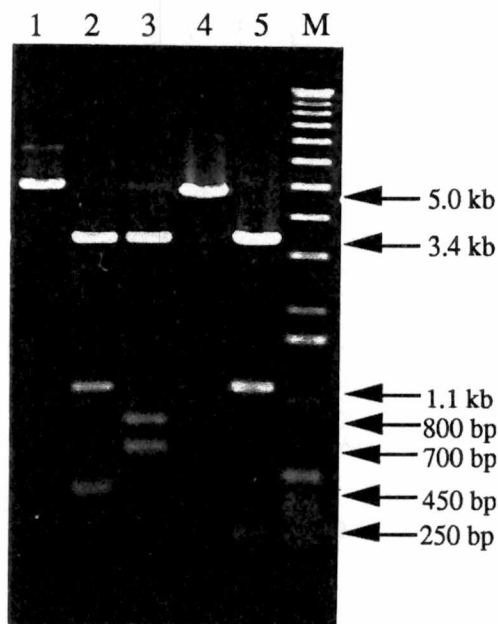


Figure 4.4. Digestion mapping of the *Ljenod40* plasmid clones. Lanes 1-3 were from clone 38 and lanes 4-5 from clone 57. Linearization of plasmids by *Eco*RI (lanes 1 and 4) and *Bam*HI (data not shown) digestion indicated that one *Eco*RI site was lost. Double digestion with *Bam*HI plus *Hind*III (lanes 2 and 5) or *Xba*I (lane 3) showed the insert of clone 38 have 2 sites of each of the *Hind*III and *Xba*I. It also showed that clone 57 is about 250 bp short in the 3' end than clone 38.

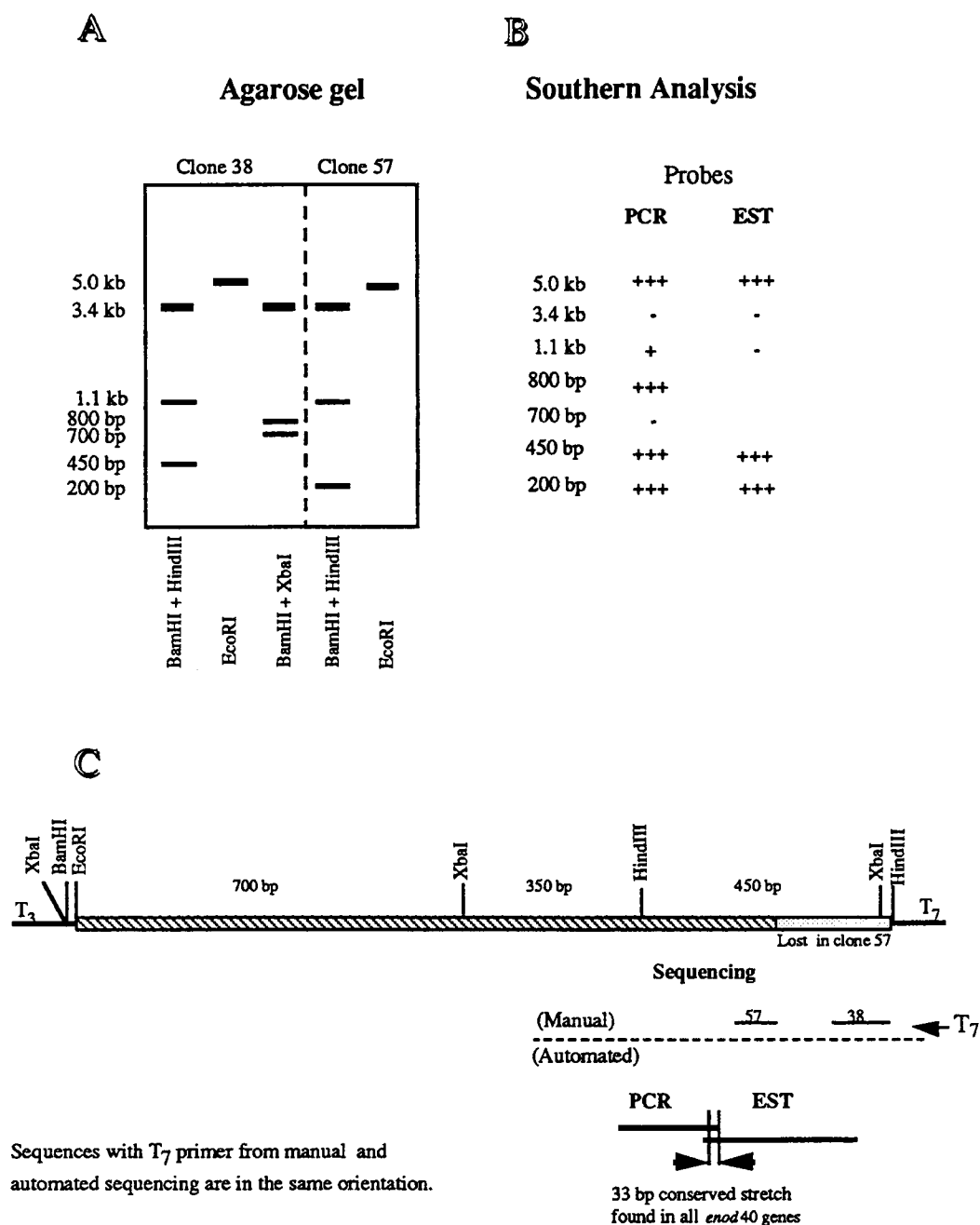


Figure 4.5. A schematic presentation of *Ljenod40* clone characterization. **A**, Diagram of the result presented in Figure 4.4. **B**, Southern hybridization of **A**. PCR probe detected bands of 5.0 kb, 1.1 kb, 0.8 kb, 0.45 kb and 0.20 kb while the EST probe hybridized to fragments of 5.0 kb, 0.45 kb and 0.20 kb. This result determined the orientation of insert, that is the insert was flanked by a *EcoRI* and *BamHI* at the 5' end and a *HindIII* at its 3' end. **C**, A simplified restriction map of the *Ljenod40* gene (top). The sequencing experiments to define the missing portion of clone 57 and full nucleotide sequence were performed both manually and automatically (middle). The 2 probes used in Southern analysis overlapped in the Region II (bottom).

CHARACTERIZATION OF THE PLASMID CLONES

Because a single restriction endonuclease (*EcoRI*) was utilized, both orientations of the insert were possible. We were able to determine quickly the orientation of insert by taking advantage of using 2 probes representing different regions of the gene. When DNA of the 2 clones was cut with *EcoRI* or *BamHI*, only a single band around 5 kb was observed. The *EcoRI* and *BamHI* sites are unique sites in the 3.4 kb vector. This result indicated that one *EcoRI* site was lost, perhaps during DNA gel extraction or ligation steps. Double digestion with *BamHI* and *HindIII* gave rise to 2 bands of 1.1 kb and 0.45 kb for clone 38, and 1.1 kb and 0.2 kb for clone 57 respectively, in addition to the 3.4 kb vector DNA (Figure 4.4 and 4.5A). In Southern analysis, the PCR probe hybridized at different intensity to fragments from either clones, with the stronger signal from the smaller band. When the EST probe was used, the signal came only from the smaller fragment. Taken together, this information demonstrated unambiguously that the 1.1 kb fragment contained the promoter region while the smaller one consisted mainly of the coding region for the transcript.

A schematic presentation of the result is given in Figure 4.5. Single digestion with *HindIII* and *XbaI* also showed two restriction sites for each of these enzymes in the *enod40* gene. Both the *HindIII* sites were inside of the coding region, one of them was close to the very 3' end. Two *XbaI* sites were localized to encompass the transcript. It also suggested that the 10 kb fragment from phage DNA was sheared in different places of the 3' end in clone 38 and 57.

SEQUENCING OF THE *L. JAPONICUS ENOD40* CLONES

Manual sequencing with the T₇/T₃ or M13 primers confirmed the conclusion about the orientation of inserts. It also showed that clone 57 was indeed shorter by 250 bp in the 3' end compared with clone 38. Having verified that two clones contained the true *L. japonicus enod40* gene, sequencing was performed, mostly with the ABI automated sequencing facility (the University of Tennessee). High purity DNA isolated with the QiaQuick plasmid Spin Kit enable 700-900 (with high confidence for 400-500) bases to be read in a single sequencing run.

T3 and T7 primers share high sequence homology

The length of clone 38 was estimated from previous restriction mapping to be 1.5 - 1.6 kb. Thus one automated sequencing experiment from each end of the insert should cover the sequence of most part of the clone. When the 5' end sequencing data read from the T₃ primer was aligned with that from the T₇ primer for 3' region of the gene to search for overlapping region, unexpected high homology (75 - 80 %) spanning the entire sequencing data was detected. This phenomenon could be explained as that the insert would have arisen from a head-to-head or tail-to-tail ligation of two pieces of the same fragment, both representing the 3' region. Repeated experiments with the same primer but different DNA template (clone 57) brought about the same result. Nevertheless, it can be expected that the probability that both clones have been through a sporadic mis-ligation reaction would be extremely low. Besides, if this did happen, the EST probe should have shown hybridization with the 1.1 kb *HindIII-BamHI* fragment. Alternatively, it could be an experimental error. As the same result came from 3 separate experiments it was also unlikely that a human mistake which occurred in several independent experiments have escaped being detected by many researchers. It was deduced that such error must have been caused by homology between forward and reverse primers.

Although it was a logical deduction, demonstration of 75 % sequence similarity between T₃ and T₇ primers (Figure 4.6) was still startling. In pBluescript plasmid series T₃/T₇ primer pair are usually used for PCR amplification. Competition between primers might eliminated T₃ to occupy the T₇ priming site. In amplification for sequencing, only single primer was applied. As a consequence, the T₃ primer recognized the T₇ priming site in sequencing reaction, probably due to the conditions (salt concentration, temperature, etc.) which favored T₃ primer-T₇ priming site annealing. For further sequencing experiments internal primers with an interval of 400-500 bp were designed for both directions.

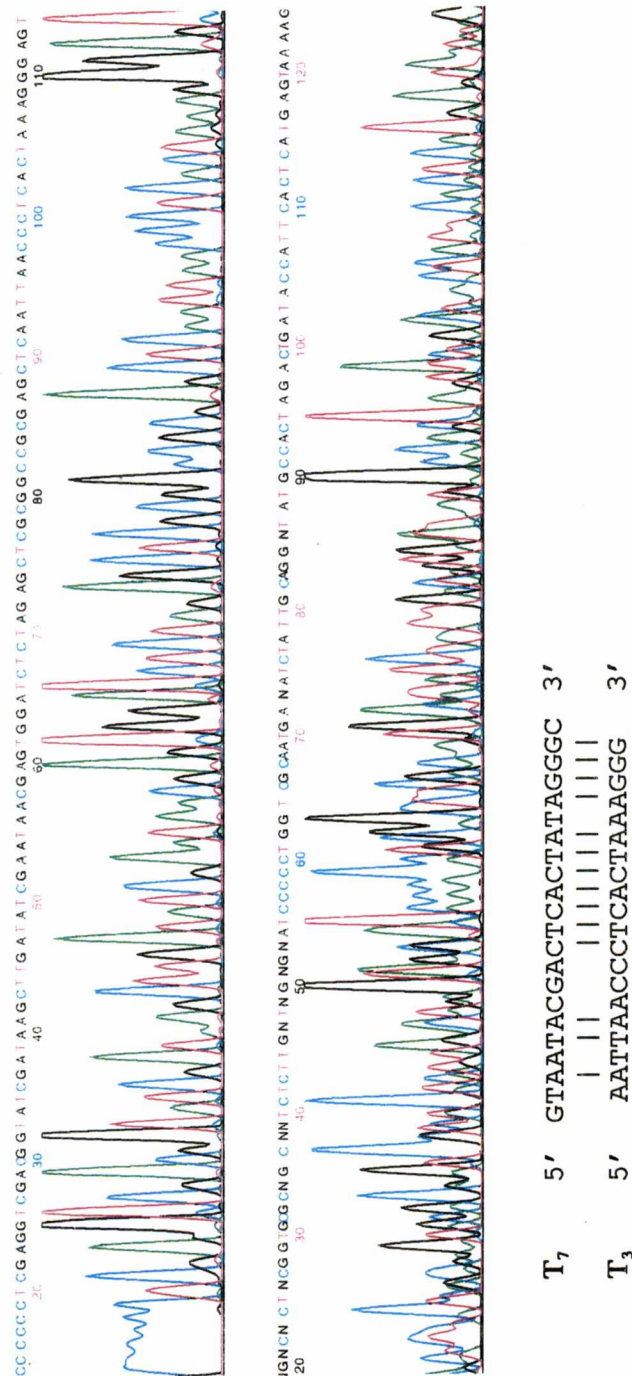


Figure 4.6. Primer T₃ and T₇ share high sequence homology. As a result, T₃ amplified DNA from T₇ site and multiple peaks are apparent in the computer print-out. Top panel: Sequence amplified with T₇ primer from the *Ljened40* clone 38B, middle panel: Sequence obtained with T₃ primer using the same template. Basewise comparison between T₃ and T₇ is given in the bottom.

SEQUENCE CHARACTERIZATION

Two groups of *enod40* gene

The *L. japonicus enod40* gene isolated from this laboratory was 1,561 nucleotides long (Appendix 1). The lengths for promoter and coding (for transcript) regions were deduced to be 759 and 792 base pairs respectively, by comparing the sequence with that of a soybean genomic clone GmENOD40 (Yang *et al.*, 1993; Kouchi and Hata, 1993). The composition of the putative TATA box (TATTTAAA), CAAT box and spacing between them are fully preserved in these two genes. All *enod40* genes identified to date possess two highly conserved regions (Figure 4.8). Region 1, located in the 5' end, contains a small ORF, while the region 2 resides in the middle of the transcript. Sequence comparison at both DNA (Figure 4.8) and peptide (Figure 4.7) levels demonstrated that they can be divided into two groups, the soybean-*Phaseolus vulgaris* group and the alfalfa group consisting of alfalfa, *Medicago truncatula*, vetch, pea and tobacco.

Plant species	Gene	Peptide
<i>Medicago truncatula</i>	-mRNA2	MKLLCWDEAIHGS
<i>M. sativa</i>	-Genomic	MKLLCWDEAIHGS
<i>Pisum sativum</i>	-mRNA	MKFLCWDEAIHGS
<i>Vicia sativa</i>	-mRNA	MKLLCWDEAIHGS
<i>Nicotiana tabacum</i>	-mRNA	M...QWDEAIHGS
<i>Glycine max</i>	-Genomic	M.ELCWLTTIHGS
<i>Phaseolus vulgaris</i>	-mRNA	MKF.CWQASIHGS
<i>Lotus japonicus</i>	-Genomic	MRF.CWQKSIHGS

Figure 4.7. Amino acid sequence alignment of ENOD40 peptides. Multiple oligopeptide sequence alignment. Two groups can be recognized. The LjENOD40 is closer to PvENOD40 (bean). The NtENOD40 (tobacco) is identical to that of alfalfa group except for short of 3 residues.

In region I, mirroring soybean-bean *enod40* genes there is a deletion of 3 nucleotides spanning codon 3 and codon 4 in *Ljenod40*. Thus, like soybean and bean the small ORF encodes a peptide of 12 amino acids. In contrast, a 13-amino acid peptide was predicted from the *enod40* genes in the alfalfa group. In region II, *Ljenod40* does not have an additional base A presented in all clones of soybean group, whereas it shares 3 out 4 bases CCCCA in a pentamer (nt 1176 - 1180) dividing soybean (CTCCA) and alfalfa (TCCTT) groups (Figure 4.8). The division of 2 groups is even more apparent in a region

Region I						
	851				900 /1051	1060
Ms-m1	aataaggatg	aagcttcttt	gttggcaaaa	atcaatccat	ggttctt--a	/attat-----
Mt-m2						/.....-----
Mt-m1						/.....-----
Ms-G						/.....-----
Ms-m2						/.....-----
Ps-m	.t...ca...					/...g.-----
Vs-m	.t..gca...					/...g.-----
Lj-G	GT..GAC...	.G.T---		...C.....	GA.	/A.T.GAAAC
Gm-G	gg.g..c...	g...---...	tc.c	.a.c.....	ga.	/ga...-----
Gm-m1	gg.g..c...	g...---...	tc.c	.a.c.....	ga.	/ga...-----
Pv-m	gg.g..c...	---...	gc		-a.	/ga...-----
Nt-m		.ctagct.g.	c.caag..c.	cact.act..	cag.t.caa.	/t.c..tgcta
	1061					1120
Ms-m1	-----gc	ttttttctga	gtagcagaag	caaataatta	agtatttttc	tccaaaggat
Mt-m2	-----..					
Mt-m1	-----..					
Ms-G	-----..				..C.....	
Ms-m2	-----..				..C.....	
Ps-m	-----..				..C.....	..tc..a...
Vs-m	-----..				..C.....	..ta.....
Lj-G	CCGAGTTA.T	AA.CAC.G.G	CATA.C..GA	AGG.GTC..T	.TG.G.AGAA	GAAG...AGA
Gm-G	-----	-----a	catt.tccga	cgg.g----	-----	-----
Gm-m1	-----	-----a	catt.tccga	cgg.g----	-----	-----
Pv-m	-----	-----g	catt.tccaa	agg.t----	-----	-----
Nt-m	gaattcca.a	aaa.gcagc.	aa.aag...a	gtgt.t..gg	.t.ga.aa..	a.actaat.g
Region II						
	1121					1180
Ms-m1	cagaagct-t	ttgttatagc	atggcaaacc	ggcaagtcac	-aaaaaggcaa	tggattcctt
Mt-m2						
Mt-m1						
Ms-G						
Ms-m2						
Ps-m		.g....t..				
Vs-m		.g....				
Lj-G	G.....	.G.C..C...	C...G...		..G.....	A...-C..CA
Gm-G	g...g..-	.g.C..C...	C.....		a.....	...-ac...a
Gm-m1	g...g..-	.g.C..C...	C.....		a.....	...-ac...a
Pv-m	a...g....	.g.C..C.ct	ca.....		-.....	...-ac...a
Nt-m	c.a.g.a.g.	.g.ca.a...	g....		-g...C.....	c...gt

* dots are bases identical to the alfalfa *enod40* gene (Ms-m1) sequence.
lines are introduced to filled the gap and to optimize the alignment.

Figure 4.8. Multiple nucleotide sequence alignment showing the similarity and differences among all published *enod40* clones. The division of two *enod40* groups were based on sequence comparisons in both amino acid and DNA levels. Abbreviation of clones: Ms-m1, alfalfa cDNA 1; Ms-m2, alfalfa cDNA 2; Mt-m1, *Medicago truncatula* cDNA 1; Mt-m2, *Medicago truncatula* cDNA 2; Gm-G, soybean genomic; Gm-m1, soybean cDNA 1; Ps-m1, pea cDNA; Pv-m1, French bean cDNA; Lj-G, *L. japonicus* genomic; Vs-m1, vetch cDNA; Nt-m, tobacco cDNA.

of nucleotide sequence prior to the conserved region II, where two stretches of sequences are absent in soybean and bean clones. *Ljenod40* had the full length of bases in this region, nevertheless, the homology between it and alfalfa group was significantly low and the sequence between the two missing stretches in *Ljenod40* was closer to that of soybean (Figure 4.8). I here propose to divide *enod40* genes into 2 groups and place *Ljenod40* tentatively in the soybean-*Phaseolus vulgaris* subgroup. Such division can also be seen easily in the numerical comparison of sequence similarity of all *enod40* genes (Figure 4.9).

Ljenod40 promoter

The promoter of *Ljenod40* shares about 60% sequence similarity with that of *Gmenod40* from soybean (Figure 4.10a). Immediate upstream of the putative transcription start point for about 80 base-pairs, the *Ljenod40* showed nearly 90% homology to *Gmenod40*, including a short sequence of 25 bp which is identical in the two species and encompasses two putative promoter elements, i.e., the CAAT and TATA boxes. The homology diminishes towards further upstream with a 50 % sequence identity spanning about 400 bp.

	<i>P. vulgaris</i> , mRNA	Soybean, Genomic	Soybean, mRNA-1	Soybean, mRNA-2	<i>L. japonicus</i> , Genomic	<i>M. sativa</i> , Genomic	<i>M. sativa</i> , mRNA-1	<i>M. sativa</i> , mRNA-2	<i>M. truncatula</i> , mRNA-1	<i>M. truncatula</i> , mRNA-2	<i>P. sativum</i> , mRNA	<i>V. sativum</i> , mRNA	Tobacco, mRNA
<i>P. vulgaris</i> , mRNA	1.00	0.70	0.71	0.58	0.49	0.51	0.45	0.51	0.50	0.47	0.44	0.43	0.39
Soybean, Genomic		1.00	0.97	0.97	0.44	0.40	0.43	0.42	0.41	0.44	0.39	0.39	0.39
Soybean, mRNA-1			1.00	0.92	0.51	0.47	0.43	0.44	0.47	0.40	0.41	0.40	0.38
Soybean, mRNA-2				1.00	0.49	0.45	0.36	0.40	0.45	0.35	0.36	0.37	0.35
<i>L. japonicus</i> , Genomic					1.00	0.34	0.41	0.41	0.40	0.40	0.41	0.40	0.39
<i>M. sativa</i> , Genomic						1.00	0.94	0.96	0.94	0.95	0.64	0.61	0.42
<i>M. sativa</i> , mRNA-1							1.00	0.94	0.96	0.97	0.71	0.68	0.42
<i>M. sativa</i> , mRNA-2								1.00	0.95	0.90	0.64	0.61	0.42
<i>M. truncatula</i> , mRNA-1									1.00	0.96	0.63	0.60	0.42
<i>M. truncatula</i> , mRNA-2										1.00	0.67	0.65	0.42
<i>P. sativum</i> , mRNA											1.00	0.83	0.40
<i>V. sativa</i> , mRNA												1.00	0.40
Tobacco, mRNA													1.00

Figure 4.9. Numerical presentation of *enod40* gene sequence similarity analysis. Division of 2 groups can also be made using *Ntenod40*. Because the presence of promoter and 3' region in some genomic clones, the values are lower than that of comparison among coding regions.

Figure 4.10a.

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310      320      330      340      350      360      370      380      390
Ljenod  ATGAGTTGCTTATAGATGAACAAATTTGCTCATCAATACAACACTCACATCAATTGTAGGAGTAGATTGCACAATT
Gm40.p  AAGCTGAAATTTGGCGAAGGACAAGATAGAGGTCACAAATTCAATATGAACAT--ATTATAACTAAGTACTGCTTAAAT
      1270      1280      1290      1300      1310      1320      1330      1340

400      410      420      430      440      450      460      470
Ljenod  TTGATGAGAAGTAATTGTTCTTAATAATTAAGCTAATATGTGCTTTCTAATATATTCATATGTATCAAAAACAGGTTAA
Gm40.p  TAGAAAAAACAGAAATGAGATAATCAAATTAGGTTA-----GGGATCTCAAAACTTCAT-TCCATGTAAACCA-GTAGA
      1351      1360      1370      1380      1390      1400      1410      1420

480      490      500      510      520      530      540      550
Ljenod  TAGTACAAGATTTGT-ATTAT-AT-AATTATGGTAGCT-AGGTTTGTAAAAATTTCCCATTTAAACCAGAGTGAAAAA
Gm40.p  GA----AAAATGTGTAAACAAGATCAATTAGGTCAGGTCATATTGCTGGTTAAGGTTAAGGTTAAGTGAAGTTCAAGAA
      1421      1430      1440      1450      1460      1470      1480      1490

551      560      570      580      590      600      610      620
Ljenod  AGTAAATGGGGGAATTAGGT--AGGT--AATTAGGTTAAGGTTAATATTGTCA-TATAAACTAACTAATCCTTTATTTG
Gm40.p  AGT-TAGGCCGGTATTAGGTTAAGGTTAGATTCAATAAACCAAGAAATGTTATTTTAATCCATTTAAT--TATTTTAA
      1500      1510      1520      1530      1540      1550      1560      1570

630      640      650      660      670      680      690      700
Ljenod  TTTGTTTATTTCAATGGACACTGTTCTGTATGTTATCTCTCATTACTTTCTAGAATTGCATCCTATGATAGCAAAA--
Gm40.p  TGAGCGCAGTACAATTTTCTTTCTTGAGTTTCTATATTGCA--ACTTTTGATGATAGTTTTTTA-AATGGAAAAAAG
      1580      1590      1600      1610      1620      1630      1640

710      720      730      740      750      760      770
Ljenod  GCATTTGAATAATGAATAGGTGATG---CCATTTCTGAGTTCTCAATCATCAACTATTTAAAGCAATGATCAGAAACT
Gm40.p  GCAAAATGGATAATGAAAAGATGATAGTACTTCTTAGTTCTCAATCATCAACTATTTAAACAATGGTCAGAGGCT
      1660      1670      1680      1690      1700      1710      1720

780      790      800      810      820      830      840
Ljenod  TTCTCCACTAGCTTCC----CAGAGAGCCATTGGGGGAAAACCGAACGGACAAGTCTATC----CAAGGTGAATCTG
Gm40.p  TTCTCCACTAGTTTCTGTGTAGAGAGCTCTTT-GGACAACACCCTCTAAACCAATCTATCAAGTCCTGATTAATCTG
      1730      1740      1750      1760      1770      1780      1790      1800

850      860      870      880      890      900      910      920
Ljenod  AGACATGAGATTTTGTGGCAAAAATCCATCCATGGCTCTTGAAGAAGCATGGAGAGGAATGTGTGAGCGGGTCAGGG
Gm40.p  GAGCATGGAGCTTTGTGGCTCACAACCATCCATGGCTCTTGAAGAAGCTTGGAGAGAAAGGGGTGTGAGAGGAGAGG
      1820      1830      1840      1850      1860      1870      1880

930      940      950      960      970      980      990      1000
Ljenod  TTTTCACCCCTCACACACATCCTATGCATCTTACAGTTTGTGTTAGGCTTAGCCATTGGCTTCTCTAGTCTTCAAAA
Gm40.p  TGCTCACTCCTCACAC----TCCCTCACTTAAACAGTTTGTGTTGGCTTAG-CTTGGCTTCTCTGATCAACAAGG
      1890      1900      1910      1920      1930      1940      1950      1960

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Figure 4.10. Base-wise sequence comparison of *Lotus* and soybean *enod40* genomic gene promoter (a) and coding region (b). The *Ljenod40* is completely the same as *Gmenod40* in the putative TATA-CAAT region. Based on this the transcription startpoint was deduced. The *Ljenod40* promoter is also highly rich in As and Ts. I: Promoter region (59.9% identity in 731 bp overlap). II: Coding region (73.8% identity in 397 bp overlap).

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      1090      1100      1110      1120      1130      1140
Ljenod AGGAGTCTTTATGAGTAGAAGAAGTTGAGAGAGAAGCTTTGGCTACAGCCTGGCGAACCG
      ||||| || |||||
Gm40.c GCGGAACGAGATACACATTCTCCGACGGAGGAGAGGC-TTGGCTACAGCCTGGCAAACCG
      1990      2000      2010      2020      2030      2040

      1150      1160      1170      1180      1190      1200
Ljenod GCAAGTCAC-AGAAAGGCAATGGACCCATTAGGGGTTCCTTATGGCTATGTATGAATGAC
      ||||| || |||||
Gm40.c GCAAGTCACAAAAAGGCAATGGACTCCATT-GGGGTCTCTATGGCTATGT---AGTG-C
      2050      2060      2070      2080      2090      2100

      1210      1220      1230      1240      1250      1259
Ljenod TCTTTTGTAGTTCTTCTTCAAGTAGAATGTAATAAACAAAAATGTTCTTCTTCCTTT---
      ||||| || |||||
Gm40.c TCMRATGTAGTTCTTCTTGTCTGTAGAATGTAATAATAAACAAAGTTGGTCTTCCCTTTTGA
      2110      2120      2130      2140      2150      2160

      1260      1270      1280      1290      1300      1310
Ljenod GAGGT-----GGTGTT-CATTC--ATATCACCCAATTTCAGCTGACTAGACTCCAGTTT
      ||||| || |||||
Gm40.c GAAGTTACCAGCTTTTGTCTGTCAAAATTACTCAATTTCAGCTGACTAGAAATCCCTTTC
      2170      2180      2190      2100      2210      2220

      1320      1330      1340      1350      1360      1370
Ljenod GCTCTTCAGTTTCTGCAGATGAGTAGGTAGGTAA-TTATGATCACCTTGTCTCTTCTTTT
      ||||| || |||||
Gm40.c TCTCTTCAGTTTCTGCAGATGAGTAGGTAGGCAATTGTGATCAC--TCCCTTCCCTTTT
      2230      2240      2250      2260      2270

      1380      1390      1400      1410      1420
Ljenod TCTGT-GTCTGTTTTCTTTCTCATGTGTGTATG-GTTGTTGTCTTGTGGCCTTAT-AT
      ||||| || |||||
Gm40.c CATGTCTTCTGTGTTCCCTTTTCCATGCTTGTGTTGTGTT-AGTTATGACCTTATGAG
      2280      2290      2300      2310      2320      2330

      1430      1440      1450      1460      1470      1480
Ljenod GCAATAATAGTATTTGAGTGATGTTTTTGTTCCTTCAAGTGTAGGATTGTGTTTGTAGA
      ||||| || |||||
Gm40.c GAAATAAAGAAT---AGTACAATTCTAGTCCCT--CAGTTTAGGATTGTATTCTATTGA
      2340      2350      2360      2370      2380      2390

      1490      1500      1510      1520      1530      1540
Ljenod AGTTGATCGAGTCGACTCCCTTTTAGTGAGGGTTAATTGAGCTCGCGCCGCGAGCTCTAG
      |||||
Gm40.c ACTTTATTAGAAAAGTTTCCAGAGTCCTTTCTAAGGTTGGAGTCGAATAATGTTTCATGAT
      2400      2410      2420      2430      2440      2450

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Figure 4.10b. (continued).

Discussion

The *enod40* was entitled "the most fascinating plant gene" by Brewin and Legocki (1996). The expression pattern of this gene, which was induced quickly in root pericycle and dividing root cortical cells by rhizobia infection or Nod factor treatment, implied that it could be of potentially great importance in organ development. The dividing cortical cells induced by rhizobia are destined for nitrogen-fixing nodule formation. The root pericycle is the tissue where lateral roots and the nodule vascular system originate. Neo-organogenesis is involved in both cases. Expression of *enod40* precedes cell division in these cells implies that it may play a role in regulation of the cell cycle. In transgenic plants, cell division was induced by overexpressing *enod40* gene (Yang *et al.*, 1996). Interest in this gene was further stimulated by the finding that *enod40* possibly participates in plant hormone perception and signal transduction. In addition to soybean, bean, pea, alfalfa, vetch and tobacco genes, a cDNA clone was recently obtained from *Sesbania* (Corich *et al.*, 1997) with a high sequence homology to *Ljenod40*.

From soybean, alfalfa and *M. truncatula* two copies of the *enod40* gene have been cloned (Yang *et al.*, 1993; Kouchi *et al.*, 1993; Crespi *et al.*, 1994; Asad *et al.*, 1994). The *L. japonicus enod40* genomic clones have been almost simultaneously isolated from three independent laboratories (Flemetakis *et al.*, 1997; H. Kouchi, personal communication, and this study) reflecting a growing acceptance of this plant as a model legume as well as a large scientific attraction to this gene. Both Flemetakis' and Kouchi's groups isolated two genes from *L. japonicus*. While using the EST clone provided by Dr. F. de Bruijn at Michigan State University, Qunyi Jiang (Racheff Chair of Plant Molecular Genetics, the University of Tennessee) detected a single copy of *enod40* in 'Gifu' genome (unpublished data). Failure to find the second *enod40* gene in *L. japonicus* could be caused by the choice of the EST clone as probe, which represents the 3' half of the gene. The 3' region of the *enod40* from different plants showed much less sequence similarity compared with the 5' half containing the two highly conserved regions. The two genes in *L. japonicus* reported by Flemetakis *et al.* (1997) share overall 60% sequence homology. It was likely that the second gene escaped being detected by the EST probe due to low sequence homology or high stringency in Southern hybridization.

When *enod40* was closely examined for gene structure, the absence of introns, the characteristics of transcript/cDNA and ORF, a dilemma is apparent. The RNA structure analysis indicated that the *enod40* transcript is very stable (Crespi *et al.*, 1994). Many isolated cDNA clones have polyadenylated 3' tails (polyA). Thus the transcript upstream of polyA was considered as a coding region or mRNA by many authors in their description of sequences when submitting to databases. On the other hand, recognition of the short ORF poses a contradiction to the definition of mRNA as a polypeptide-coding ribonucleotide sequence. Of the two conserved regions only the region I contains the small ORF. Crespi *et al.*, (1994) labeled region II as 3' untranslated region (3' UTR) and showed altered phenotypes in transgenic plant transformed the 3' UTR independently or in combination with the ORF. Taken the sequence feature and its biological activity together, they proposed *enod40* functioning as a riboregulator. A similar result was reported by Jeff Schell's group (Schell, 1997) who identified a second ORF in region II encoding a peptide of 16 amino acids and observed alteration in transformant plant carrying a region II sequence. An ORF equivalent to the one reported by Schell's group with comparable peptide product was not obvious in all *enod40* genes. Using the same approaches by Kondorosi's and Schell's groups, however, phenotypic changes were not observed by other laboratories (H. Kouchi, personal communication).

The short peptide deduced from the putative ORF in Region I are of 12 -13 amino acids long (except for NtENO40 which is 10 amino acids long, Figure 4.9). Combined with the gene expression pattern and cellular response (cell division induction, change of hormone perception), it was proposed that the short peptide might function as a novel type of plant hormone (van de Sande *et al.*, 1996). Peptidyl hormones are common in animals. In plants the only known peptidyl molecule with hormonal characteristics is systemin (McGurl *et al.*, 1992), an 18 amino-acid peptide inducer of proteinase inhibitor biosynthesis in response to wounding and pest/pathogen attack. Although the ENOD40 peptide was detected in transgenic tobacco cells by monoclonal antibody (van de Sande *et al.*, 1996), the purification of a protein product has proven to be far more difficult and has not been demonstrated yet.

Region II is even more conserved among all isolated *enod40* genes compared with region I. This region is also longer than region I. Although the ends of this region has not yet been defined, multiple sequences alignment (Figure 4.8) showed a length of 60 bp that is highly conserved among all clones. Within it there is an invariant 30 nucleotide stretch. Such high homology of non-coding sequence (putatively) has not been reported in other genes. The discussion of whether the *enod40* product is a riboregulator or novel plant hormone is not set yet and extensive tests and experimentation are necessary. Regardless the plant hormone hypothesis for *enod40* product, which has to be backed by purification of the short peptide and demonstration of hormonal properties, the high conservation of region II strongly suggests a biological function. As no conserved ORF like the one found in region I has been identified, it would be logical to speculate that *enod40*, at least the region II may exert its activity via its transcript.

CLASSIFICATION OF *LJENOD40*

Ljenod40 was tentatively placed in the soybean-French bean group. The classification was based on peptide sequence and partial DNA sequence data, especially in region I and II. The entire sequence, however, is quite different from that of both soybean and alfalfa groups. The short peptide of ENOD40 in all members of alfalfa group (alfalfa, *M. truncatula*, pea, vetch), except tobacco, is identical. The non-legume NtENOD40 differs in three missing amino acids (aa 3 - 5) and one substituted residue. Otherwise it is perfectly the same as MsENOD40. The LjENOD40 is closer to the PvENOD40 of bean in that there are only two amino acid differences in the variable residues. At the nucleotide level, like the soybean and bean *enod40*, *Ljenod40* has three bases missing in nt 865 - 867 and two additional bases in 898 and 899, which are absent in members of alfalfa group. In the region from 1069 - 1120, there are two missing stretches of 11 and 25 bases, respectively, in both bean and soybean *enod40*. *Lotus* and tobacco genes, however, have both of these stretches but without apparent sequence similarity to that of the alfalfa group, which is identical among the members (Figure 4.8). In the 16 base pairs flanked by the two stretches, *Ljenod40* shared 7 bases with soybean, 2 with alfalfa, 1 with both and 4 with none of the groups.

Figure 4.9 is a computer-generated digital similarity comparison of all *enod40* sequences published up to September, 1997. The value will be lower when a genomic

clone was compared with another genomic or cDNA ones. For example, a 500 bp promoter region was 60 % homologous to the same region of the soybean promoter and a 73.7 % similarity was obtained between the *Lotus* and soybean *enod40* coding region spanning 400 bp, the overall homology between the 2 sequences was only 44 % because of the further 5' promoter region and 3' non-coding (with regard to the transcript) region. Although the value of homology between *Lotus* and other members of soybean group is substantially lower compared with the one between soybean and bean, it is still higher when compared with members of the alfalfa group. Such division can also be visualized via use of tobacco *enod40*, which divides the two groups by a cut-off similarity of 40 % (Figure 4.9). Although the difference between the two groups may not significant, it provides a supportive hint. This division is more convincing when the peptide sequences are examined (Figure 4.7).

LJENOD40 PROMOTER

TATA and CAAT boxes and promoter strength

The putative *Ljenod40* promoter is about 760 bp long. The TATA (TATTTAAA) and CAAT boxes were located in the - 40 and - 51 regions with respect to the putative transcription start point. The two consensus regions are sometimes referred as - 30 and - 80 elements due to their positions in many eukaryotic promoters (Levine, 1990). The TATA element is the site for multi-functional transcription factor TFIID binding and critical for accurate transcription start while the CAAT is an enhancer element. Binding of the CAAT box by other transcription factors facilitates assembly of transcription machinery consisting of RNA polymerase II (Pol II), TFIID, and accessory protein factors at the core promoter (TATA box and Initiation element, Inr, encompassing the transcription start point) through a recruitment mechanism. Though the 4-base consensus of CAAT is intact, none of the flanking bases commonly associated with it was found in *Ljenod40*. The genuineness of CAAT element was further challenged by the unusual short spacing (11 bases) between the TATA and it. Searching further the 5' region revealed no second CAAT sequence, instead, a CCATT preceded by a G was located 15 bases upstream of CAAT. It is possible this sequence serves as an alternative enhancer element.

In eukaryotic genes, the promoter (5') region was divided into core promoter about 50 bp upstream of the transcription start point and further 5' enhancer region (Goodrich *et*

al., 1996; Novina and Roy, 1996). The core promoter contains the TATA box and a less conserved initiator element (Inr) spanning the transcription start site. In plants more than 50 % of promoters do not possess both TATA and CAAT elements. Some promoters don't even have any of these elements (null promoters). The presence of one or two, or absence of promoter consensus elements is correlated with the strength of a promoter. The *Ljenod40* promoter is highly rich in As and Ts. These two bases contribute 67.5 % of the 759 region including 72.5 % immediate upstream of the putative TATA box. AT rich sequences are the sites for binding of regulatory proteins AT-rich elements (ATREs), homologues of high mobility group genes (HMGs, Guilfoyle, 1997). The putative TATA box significantly resembles the 8-base consensus ATRE binding site (TATTTWAT, where W can be A or T) found in many late nodulin genes as well as other dicotyledonous plant genes (Forde, 1994). The only difference between the proposed TATA box and the ATRE binding consensus was the location. The 8-base ATRE binding sequences are usually found at -150 or farther away. Therefore it is not clear whether or not the TATTTAAA sequence in *Ljenod40* is a genuine TATA box or a ATRE binding site. Regardless the role of the octamer, the high AT content in *Ljenod40* promoter may render the gene high inducibility. In legumes as well as non-legumes it is likely that the *enod40* gene, like nodulation *per se*, is not essential for plant growth. The characteristics of this gene, its expression pattern, hormone-like activities and possible participation in hormone perception/signaling are all consistent with the idea that *enod40* promoter might not be a strong promoter but is responsive to induction, as suggested by the richness of A and T bases. In such circumstances, recruitment of RNA polymerase II, induction and specificity of expression may depend on tissue- or developmental-specific transcription factors (Ptashne and Gann, 1997; Carey, 1994; Meisel and Lam, 1997).

L. japonicus is accepted rapidly as a model legume. The increased interest in this plant is evidenced by the large proportion as well as volume of posters and speeches presented in the 11th International Conference on Symbiotic Nitrogen-Fixation (Paris, July 18-24). Isolation of *Ljenod40* will surely help to promote use of this model system. Study of the regulation of hormone balance by *enod40* in induction of nodulation will be facilitated by homologous system. Currently construction of chimeric expression vectors (constitutive sense gene expression under CaMV 35S promoter control, antisense, *Ljenod40* promoter directed β -glucuronidase reporter gene, etc.) are under way. S1 mapping to pinpoint the transcriptional site will be performed soon in this laboratory.

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Part V

**A SYNTHESIS OF IDEAS:
DRAWING OBSERVATIONS TOGETHER**

Throughout the project, the involvement of plant hormones in organ development frequently surfaced. Induction of cell division in the root pericycle and cortical cells during nodule initiation is clearly hormone-related. Hairy root formation was attributed to *A. rhizogenes* *rol* genes, which have been shown to cause increased sensitivity to plant hormones, particularly, auxin in plant cells (Maurel *et al.*, 1994; Capone *et al.*, 1989). When the transgenic hairy roots were used to study nodule organogenesis different types of hormone regulation conflict, which could result abnormal phenotype, as implied in the formation of soybean meristematic nodules. Plant hormones are critical ingredients of media for tissue/cell culture and regeneration. The spontaneous regeneration of *L. japonicus* transgenic plants from AR12-induced hairy root culture reported in this thesis and by Chian *et al.*, (1998) was independent of exogenously applied auxin and cytokinins, indicating a fundamental change of a still unknown regulatory mechanism switching cell/tissue differentiation from a hormone-dependent to hormone-independent status. Hormone-free regeneration of *Lotus* plants was also reported by Oger *et al.* (1996); nevertheless, these regenerants emerged from the cotyledon-attachment site of established plants, and can be viewed as new shoots. *enod40* caught our attention because of its potential regulatory role in nodule development. Modulation of *enod40* activity has been shown to confer auxin tolerance to cell culture and trigger tissues/cells to enter cell cycle under conditions which would not allow it to happen normally in the absence of the gene or gene product (Crespi *et al.*, 1997; van de Sande *et al.*, 1996).

Hormone perception, signaling, and their mode of action have been targeted for investigations in several fields of plant research such as plant growth, embryogenesis, flower and root development, etc. Attempts were also made to illustrate the role of plant hormones in symbiotic nitrogen fixation and nodulation. In the following paragraphs I will try to integrate information (existing knowledge, experimental results by other researchers, theories and hypotheses) into model(s) and to align them with our understandings and observations concerning nodule development. These thoughts, however, are incomplete. The purpose is to stimulate critical thinking.

Control of nodulation by plant hormones

AUTOREGULATION AND AUXIN BURST CONTROL (ABC).

It was originally observed that the number of nodules formed by legume-*Rhizobium/Bradyrhizobium* interactions was more or less constant on a root of given plant (Nutman, 1952). Excision of mature nodules allows formation of new nodules on soybean roots without re-inoculation of rhizobia (Caetano-Anollés and Gresshoff, 1991). This phenomenon of developed nodules suppressing emergence of new nodules is controlled by a mechanism referred as autoregulation or feedback inhibition (Caetano-Anollés and Gresshoff, 1990; Takats, 1990). In soybean these new nodules formed in the zone of original infection while in alfalfa nodules developed from regions which are sensitive to rhizobial inoculum (Gresshoff, 1993). It seemed that the regulation of nodule number was executed by plants. For example, in wild-type Bragg and Peking inoculated by the same *Bradyrhizobium japonicum* strain USDA110, the former produces in average 30-40 nodules while the latter makes only 10 -15. A hypothesis was proposed by Gresshoff and his colleagues (Delves *et al.*, 1986), which stated that infection by *Rhizobium/Bradyrhizobium* triggers production of a signal molecule from roots called the Root Synthesized Activator (RSA). The RSA is then translocated to shoot and induced a second signal termed the Shoot Derived Inhibitor (SDI). This SDI is in turn feedback to roots and suppresses formation of new nodules. To date, none of the signal substances has been identified.

The systemic nature of inhibition was confirmed by split root experiments where inoculation of rhizobia on the two halves of root system were separated temporally and spatially, and reciprocal grafting where shoots and roots of different nodulation type were grafted together. These experiments showed clearly that the autoregulation of nodulation phenotype was controlled by scion (shoot) but not the stock (root). It was further demonstrated by differential removal of different shoot parts that the leaves but not apical meristem are most likely the source of controlling factors (Delves *et al.*, 1992).

This model was expanded as the Auxin Burst Control (ABC) to account for autoregulation by changes in the internal free auxin level (Gresshoff, 1993). In the expanded model, the signal that induces SDI was thought to be cortical cell division and the

SDI *per se* could be an enhanced level of auxin (or auxin-like activity). Once cortical cells start to divide, to various extents, nodule meristems form inside nodule primordia and become cytokinin autonomous. Alternatively, unloading/depletion of hormone (auxin) or other substances from phloem can generate a "debt message" (Jackson, 1997). Either of these changes can signal a perturbation in pre-existing auxin-cytokinin balance and send a pulse to shoot, which triggers the autoregulation/ABC mechanism. Responding to this signal, leaves begin to accelerate auxin biosynthesis (an auxin burst) and "pump" it down through the phloem to the roots. High level of auxin is suppressive to further cell divisions and nodule growth.

The decision on which nodules are to be arrested and which are passed is probably made by measuring the nodule developmental state. Nodule primordia that already passed through a critical growth stage (threshold) will continue to develop and form mature nodules while others still below the threshold will be suppressed.

The assumption that cell division is the signal for autoregulation was based on approach-grafting experiments (Caetano-Anollés and Gresshoff, 1990), in which plants of different nodulation phenotypes were grafted together with their root systems separated. The critical information comes from two non-allelic nod⁻ mutants, nod49 and nod139 (Mathews *et al.*, 1989). In nod49, cortical cell division can be induced but the division loci cannot develop into nodules. In nod139, on the other hand, no cell division activity can be detected. When wild-type Bragg or nod49 were grafted to Bragg plants as reporter roots, inoculation on one side first suppressed nodulation on reporter roots. However, when nod139 was grafted to Bragg plants, no suppression occurred. These results showed that cortical cell divisions are a prerequisite for the ABC function. It was deduced from these experiments and reciprocal grafting results that the *nts* mutants were impaired in their ABC mechanism. No or very lower levels of auxin burst is presumed in these plants; therefore, no or little nodulation suppression occurs. Nodule primordia formed on roots of *nts* plants proceed to mature nodules, although some arrested loci remain (Caetano-Anollés and Gresshoff, 1991). This suggests the existence of a localized suppression mechanism other than the systemic (*nts*) suppression system.

Regrowth of nodule primordia in the original nodulation window by excision of existing nodules or nodule meristems in tropical legumes producing determinate nodules

was likely to be achieved by releasing the "apical" dominance and changing the direction of metabolites influx. In agreement with this is the finding that excision of lateral root tips and nodule meristems induced more and fast nodule formation. In alfalfa and probably other temperate legumes, the nodule meristems may also contribute to the inhibition of potential cortical cell division loci through apical dominance. Suppression by autoregulation is mainly on the formation of new cortical cell division centers.

Auxin as a component of autoregulation is supported by an experiment in which treatment (injection) of soybean stem with IAA severely lowered nodulation (Olsson, 1988; Gresshoff and Caetano-Anollés, 1992). Direct evidence of auxin involvement in autoregulation is not yet available. It is hoped that isolation of the supernodulation *nts* gene would help to clarify the mode of action of regulation.

The ABC mechanism of autoregulation is probably to operate only in a short term. When new cortical cell division activity ceases, the ABC diminishes. Control of nodule mass is likely to be executed by another mechanism, the metabolite flow. It is known that in plants the shift of sink-source relationship in seed-setting and flowering stages inhibits vegetative growth and development in other organs. The switch of such relationship is also made by plant in response to environmental stimuli, such as pathogen infection (Herbers *et al.*, 1996). In legumes, it was sometimes observed that when fewer than normal nodules were produced, they were unusually large. It appears that developing nodules which escaped from autoregulation inhibition establish themselves as sink and dictate nutrient flow. Excision of mature nodules enables reorientation of metabolite influx. Similarly, when higher concentration of inoculum was used, the *nod49* mutant which normally does not nodulate, produces one or two large nodules (J. Blauenfeldt, University of Tennessee, unpublished). The source to sink metabolite flow is probably activated by a signal from *Rhizobium* infection/Nod factor treatment not related to cell division.

POSITIONAL INFORMATION IN NODULATION

In roots all cells are derived from the root meristem (Goldberg *et al.*, 1994; Jürgens, 1995). Cell lineage can be easily traced by anatomical as well as cellular and molecular biological approaches (Sheres *et al.*, 1996). Positional information plays a pivotal role in root tissue differentiation and growth (Meyerowitz, 1997; van den Berg *et*

al., 1995; Benfey and Schiefelbein, 1994). The destiny of individual cell or cell layer directed by positional information is via constant changes in the species and abundance of transcription factors inside the cells, indicating a programmed regulation through control of gene expressions. How the positional information can affect legume-*Rhizobium* symbiotic interaction is not known yet. The nodulation window (Bauer, 1981) which occurs in a specific region of growing roots (young root hair zone II), however, indicates its participation. The suitability of young root hairs for bacterial colonization and invasion may not solely account for the specificity of the formation of nodulation window. Indeed, susceptibility of cortical cells in younger regions to induction of cell division and formation of preinfection bridges could be controlled by positional information, as suggested by contrasting location of cell division in the two nodulation types of legumes (inner vs. outer cortex).

It is truly tempting to speculate on the role of plant hormones in positional information. Are changes in hormone status the effect or a consequence? The horizontal distribution of auxin, cytokinins and gibberellins in roots has long been studied and connected to nodule induction (Libbenga *et al.*, 1973). The involvement of plant hormones in positional information with regard to symbiotic nodulation is being explored (Heidstra *et al.*, 1997; van Spronsen *et al.*, 1995). Gradients of auxin, cytokinin and ethylene can be part of the mechanisms determining the location of nodule induction. For example, a higher concentration of ethylene was found in the inner cortex opposite to the protophloem cells (Spaink, 1997). This could partly explain why cell division occurred always in loci opposite protoxylem pole. A *M. truncatula* ethylene-insensitive mutant exhibited ten times more nodules (Pemmetsa and Cook, 1997). *Rhizobium* infection by point inoculation showed that cell division does not initiate in a longitudinally widespread zone of the cortex adjacent to the protoxylem pole. This observation implies that 1.) cortical cell division responds to a very narrow range of auxin-cytokinin/uridine-ethylene ratio, or other regulatory factors, and 2.) whether or not the Nod factor signaling is through diffusion or cytoskeleton networking, there is a distinct vertical distribution of effective hormone level/factors as the loci of cell division are precisely defined.

Nod factor signaling and cortical cell differentiation (cell division vs. phragmoplast formation).

CAN NOD FACTORS BE THE SIGNAL MOLECULES FOR THE MAPK PATHWAY?

The Nod factors are chitin-type lipo-oligosaccharides produced by *Rhizobium/Bradyrhizobium* nod genes. Modifications on the reducing and non-reducing terminal sugars determine their activity and host specificity (Spaink, 1992). It has been widely demonstrated that the Nod factors alone, without the presence of bacterium and infection events can induce root hair membrane depolarization (Ehrhardt *et al.*, 1992), cell division in protoplast culture as well as nodules on host roots (Truchet *et al.*, 1991; Röhrig, *et al.*, 1995; Kondorosi *et al.*, 1993; Spaink, 1992) and early nodulin gene expression. In such empty nodules several early nodulin genes including *enod2*, *enod12* and *enod40* are expressed. The spatially and temporally separated effects elicited by Nod factor treatment indicate that Nod factor signaling is less likely to involve a diffusion mechanism (Geurts and Franssen, 1996). Nod factor treatment also activated several enzymes involved in flavone compound biosyntheses such as PAL, CHS (Recourt *et al.*, 1992) and isoflavone reductase (IFR). The versatility of Nod factor induction implies that it might function as a signal molecule upstream of a common signal transduction pathway. Reminiscent of this type of signaling pathway is the Ras-MAPK (stands for mitogen activated protein kinase) pathway found in all eukaryotic organisms. The Ras-MAPK pathway is versatile but conserved. Once activated by signal perception it participates in various cellular activities, including the cell division cycle. In animal systems the MAP kinase functions on the cell cycle in both G₁ phase (Hunter, 1997) and M phase (Codswell *et al.*, 1995). In G₁ phase it activates the eukaryotic transcription initiation factors which then turn on the expression of G₁ cyclins (Peeper *et al.*, 1997) and cyclin-dependent kinases (CDKs). In M phase the kinase phosphorylates microtubule-associated proteins, therefore playing a role in microtubule rearrangement (Nobes and Hall, 1995) and cell division (cytokinesis). Although the Ras-MAPK pathway hasn't been demonstrated in plant cells, several key components, i.e., MAP kinases and MAP kinase kinases have been isolated from alfalfa, pea and *Arabidopsis*, suggesting this pathway can exist in plants (Hirt, 1997). This speculation is supported by the finding that MAPK extracted from *Arabidopsis* was activated when auxin was applied prior to extraction. In addition, a homologue of animal retinoblastoma protein (Rb) has also been identified in plants (Soni *et al.*, 1995; Murray,

1997). Rb is an important regulatory protein mediating MAPK pathway and cell cycle. Mutations in this protein result in carcinogenesis.

INVOLVEMENT OF PLANT HORMONES (AUXIN AND CYTOKININ) IN CORTICAL CELL DIVISION

In plants the involvement in cell division of the plant growth regulator or phytohormone, auxin, alone or in combination with cytokinins, is well documented. Auxin was also reported to act on expression of the *cdc2* gene encoding the p34^{cdc2} kinase (Miao *et al.*, 1993), a pivotal component for regulation of cell cycle progression. The different requirement for auxin alone or in concert with cytokinin probably reflects the difference of cell types entering cell cycle. In tissues where cells are ready to divide or have the potential to enter the cell cycle such as cambium and pericycle layer where the supply of cytokinins from xylem vessels is sufficient, auxin becomes the limiting factor. In contrast, in cortical cells where the cytokinin concentration is lower due to difficulty of diffusion, both auxin and cytokinins are needed. Supporting this point of view are reports from several laboratories showing that exogenously applied cytokinins induce cortical cell division (Bauer *et al.*, 1996). Additional evidence comes from tobacco stem pith tissue culture where auxin alone can induce *cdc2* gene expression; however, the protein product is inactive, requiring a cytokinin-dependent component to induce cell division (Zhang *et al.*, 1996; Zazimalova, *et al.*, 1995). Cytokinins appear to play a role in the regulation of cell cycle progression rather than the induction of cell division. Activation of *cdc2* by auxin does not appear to be a limiting factor as the amount and activity of CDC2 was found to be more or less consistent throughout cell cycle. Recently work from Kijne's group showed cortical cell division can be induced by an extract from the stele column. The key component in the stele extract was determined to be uridine. The uridine can replace cytokinins' role in cell division induction (Smit *et al.*, 1993), therefore it perhaps functions in parallel and interchangeably with cytokinins. It is also possible that uridine modulates auxin activity via biosynthesis of auxin conjugates (Szerszen *et al.*, 1994), therefore the active auxin to cytokinin ratio.

A HYPOTHESIS

Having viewed the information mentioned above, it is natural to ask if there are connections and what are the connections among these results and observations? At present

there are few clues in nodulation studies to suggest a link. By borrowing information from other fields it was hypothesized that cortical cell division during nodule induction is controlled through different routes. The Nod factor activates nodule organogenesis by expressing G₁ phase cyclins and CDKs through a MAPK or an analogous pathway and initiating the cell cycle process. The divergence of cellular responses, especially cortical cell division, to lipo-chitinoligosaccharide signaling cascade makes Nod factors legitimate mitogens, whether or not the signal molecule was perceived extracellularly or intracellularly. Induction of *cdc2* expression and MAP kinase by auxin may or may not be involved in this pathway. However, the cell cycle machinery does not automatically proceed to completion. Regulation of cell cycle progression is exercised by components whose activation depends on a proper ratio as well as concentration of auxin and cytokinins or uridine.

An hypothesis (Figure 5.1) was developed to explain involvement of plant hormones auxin and cytokinin in autoregulation of nodulation and the difference of control between tropical and temperate legumes, as well as findings by Ton Bisseling's group. They found that *Rhizobium* Nod factor induced both inner and outer cortical cells to enter the cell cycle, but that cell division was completed only in the inner cortical cells (Yang *et al.*, 1994). This finding suggests that Nod factors do not travel down all the way from epidermis to the inner cortex.

This model proposes that a cytokinin/uridine gradient exists in the root cortex due to symplastic-apoplastic diffusion from root vascular bundle/stele. Cortical cells in tropical and temperate legumes entering cell cycle might require different cytokinin:auxin ratio. It was postulated that a lower ratio is needed for tropical and higher one for temperate legumes, respectively, in response to Nod factor signal. As such nodule initiation occurs in subepidermal/hypodermal cells in tropical legume soybean and inner cortex in temperate plants alfalfa, pea and vetch. In alfalfa, both inner and outer cortical cells are stimulated to enter cell cycle as evidenced by reorientation of microtubules/cytoskeletons (Shiboaka, 1994; Staehelin, 1994). However, progression of cell division through G₂ to M phase needs accessory factor(s) activated by high concentration of cytokinin. In the inner cortical cells close to the vascular bundle this condition is satisfied and division proceeds to cytokinesis while in the outer cortex the cytokinin-dependent factor(s) are missing and cells are arrested in G₂ phase. Rearrangement of microtubules induced by cell cycle initiation is

coincident with the formation of preinfection bridges resembling a phragmoplast (Yang *et al.*, 1994). This organization may direct vesicle-mediated deposition of lipids, membrane proteins and other components from Golgi body to form infection thread sheath during rhizobial invasion progression, and guide the infection thread into the nodule primordia.

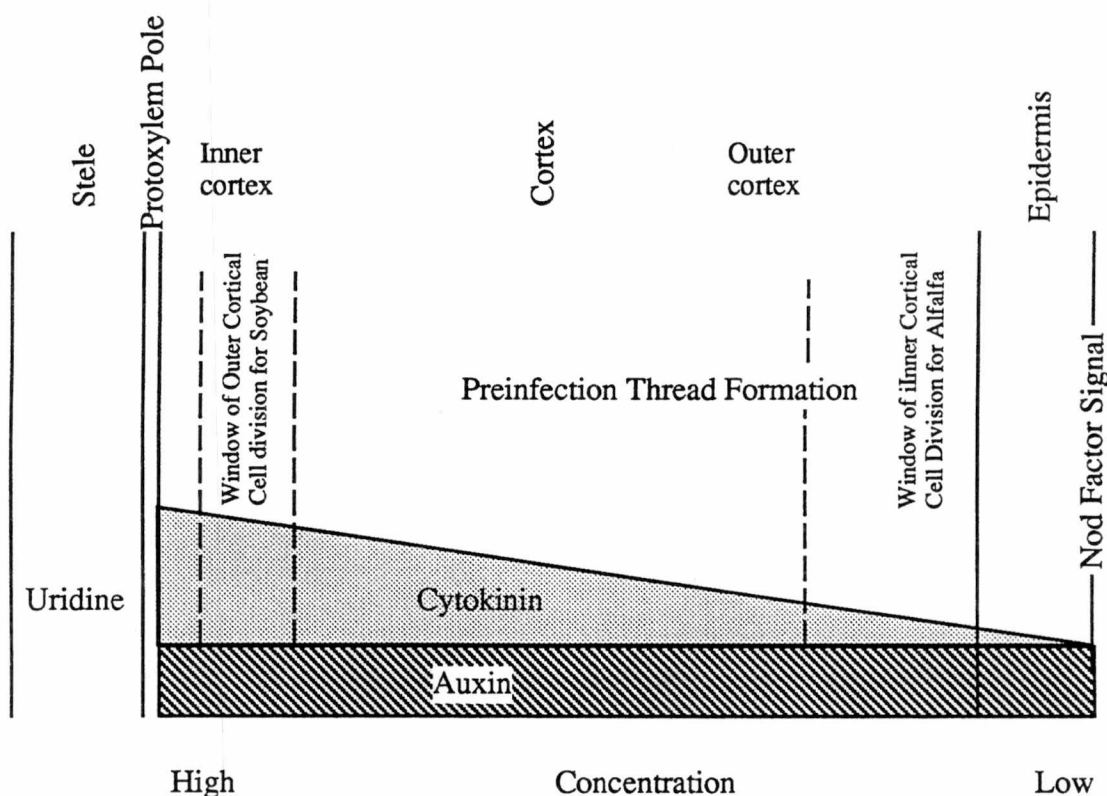


Figure 5.1. An auxin-cytokinin balance determines the inner cortical cell division and the outer cortical cell preinfection thread formation. Vascular system (includes root stele) is the main source of auxin and cytokinin, which reach cortical and epidermal cells by symplastic-apoplastic diffusion and carrier-mediated transport, establishing hormone gradients across the root. Stele factor uridine was equated here to cytokinin because it can replace the latter to exert a similar cellular response (Smit *et al.*, 1995). Legumes start nodule organogenesis in response to Nod factor signal. Different requirements for cytokinin:auxin ratio determine that cell divisions and nodule primordial formation occur in inner cortex in temperate legumes such as alfalfa and clover but in the subepidermis in tropical legumes like soybean. Nod factors also stimulate outer cortical cells entering cell cycle in temperate legumes. But these cells are arrested in G₂ phase due to lack of a phase transition factor whose expression is dependent on proper cytokinin concentration (Zhang *et al.*, 1996; Zazimalova, *et al.*, 1995). Cytoskeleton/microtubule rearrangement as a response to entry into cell cycle preconditions these cells for *Rhizobium* invasion in formation of preinfection bridge. The role of an auxin gradient/concentration in cell division is not clear and not discussed here.

The model proposed is also able to explain the distinctions in autoregulation between determinate and indeterminate nodules. It was thought that initiation of cell cycle in indeterminate legumes could have a wider range of requirement for auxin-cytokinin ratio (and perhaps hormone concentrations as well). But induction of expression of cytokinin-dependent factors occur only in a narrow window. Thus nodule development is restricted in the inner cortex close to xylem vessels. Cell division induced by rhizobial infection/Nod factor application sends a signal to the shoot and activates the autoregulation mechanism. Auxin or inhibitory substances synthesized by leaves are transported through phloem down to the roots. A high amount of auxin inhibits further cell division. The short distance between the inner cortex and vascular bundle in temperate legumes enables the inhibition to be exerted sooner. In tropical legumes, diffusion from the phloem to subepidermal cells causes a non-uniform auxin distribution reaching this region and a time lag in inhibition. Nodules already past a developmental threshold (probably measured by programmed cessation of meristematic activity) continue to grow while those under the threshold are suppressed. The resumption of growth of suppressed nodule primordia in the initial infection window by the depletion of mature nodules probably is a consequence of redirection of metabolite flow.

In Figure 5.1, the auxin concentration presented as evenly distributed throughout root tissues could be misleading. The emphasis is that the auxin level is in an equilibrium state prior to cell division induction and regulation of cell cycle progression (but not induction) is auxin-independent. It is possible that Nod factor signaling can trigger localized fluctuation of auxin concentration and induce cells enter the cell cycle. However, if the signal transduction pathway is through systems other than those utilizing diffusion mechanisms, say cytoskeleton or a secondary messenger, the difference in auxin distribution should not be a concern. Experimental evidence for both the Nod factor signal transduction pathway, including isolation of Nod factor receptors and identification of the downstream substrates of the pathway and the regulation of cell cycle progression by auxin would be highly valuable.

Presented here is an immature model. There are more speculation than fact. It is hoped that it would stimulate more thinking and investigation. Hopefully the study of hormone dynamics and responses in other plants such as *Arabidopsis* will provide some

hints and information for nodulation research, which in turn could benefit our understanding about developmental biology of plant cells in general.

Nodulation and IAA transport inhibitors

AUXIN TRANSPORT INHIBITORS IN INDUCTION OF CORTICAL CELL DIVISION

An alternative theory explaining the effect of Nod factor induction of cortical cell division is related to the flavone/isoflavone compounds (Savoure *et al.* 1994). It is observed in many cases that Nod factor treatment induced activation of enzymes important to flavone/isoflavone biosynthesis (Estabrook and Sengupta-Gopalan, 1991). These results are immediately connected to findings from Ann Hirsch's group (Hirsch *et al.*, 1989). Using auxin transport inhibitors (ATIs) N-1-(naphthyl)phthalamic acid (NPA) and 2,3,5-triiodobenzoic acid (TIBA), these researchers induced cortical cell division and nodule-like structure formation. It was therefore postulated that the Nod factor induces cell division through production of flavonoid compounds with ATI properties (Jacobs and Rubery, 1988), which in turn causes localized reduced auxin level. The decreased auxin level then signals cell division. This theory can be used to explain many phenomena. But still there are some aspects that need to be clarified. 1) That Nod factors induce or enhance biosynthesis of flavonoids/flavones with ATI properties has to be demonstrated. 2) If Nod factors induce flavonoid precursors capable of being converted to ATIs, then the pathway and time course for conversion need to be identified. 3) Cellular compartmentation of ATI compounds needs to be investigated and 4) Cytological comparison of the ATI and Nod factor-induced nodule-like structures with their peripheral region needs to be performed. In addition to the organization of vascular bundle, tissues equivalent to the infection zone and nodule parenchyma, the location of initiation of cell division and preinfection-thread-like structures has to be demonstrated.

Comparable rhizobia and Nod factors can elicit nodule formation starting from cell divisions in root pericycle and cortical cells. Nodule-like structures (pseudonodules) can also be stimulated by auxin transport inhibitors NPA and TIBA. Because key enzymes of flavonoid and isoflavonoid synthesis pathway such as chalcone synthase (CHS) and phenylalanine ammonia lyase (PAL) were also increased by Nod factor treatment or rhizobial infection, it has been speculated (based on these observations) that cell division

during nodule organogenesis was triggered by a localized low level of IAA as a consequence of interference with auxin activity by endogenous ATIs/phenol compounds (Hirsch and Fang, 1994; Wu *et al.*, 1996). In the cortical cell division loci which are destined to form nodule primordia, expression of early nodulin genes can be detected (Geurts and Franssen, 1996; Journet *et al.*, 1994; Crespi *et al.*, 1994). The *enod40* was also induced in root pericycle cells which provide cortical nodule primordia with the nodule vascular system (Rolfe and Gresshoff, 1988). The earliest detection of expression of *enod12*, *enod40* and *rip* occurs 2-6 hours after inoculation, which is far ahead of cytokinesis being observed. Thus expression of these early nodulin genes was often interpreted that these genes are involved in the regulation of nodule induction. Bearing in mind that the eukaryotic cell cycle takes on average 20 to 24 hours. Although it is not clear yet how long the cell cycle takes exactly in nodule development in legumes, it is possible that it would be not less than 16 hours. Considering the considerable complexity of nodule initiation and development, genes expressed before completion of first cell divisions should not be solely attributed to regulation of cell division. The signaling cascade of Nod factor induction should not be merely explained as an amplification of a signal. The multiple cellular responses to Nod factor treatment indicate that there could be divergent signaling pathways activated. Some of them may converge late to direct cell division, differentiation and development, while others play roles in metabolism, transport and other cellular activities, all these are essential to formation and normal functioning of nodules. The question of cause or effect remains. Right now we don't have evidence that whether *enod2*, *5*, *12*, *40*, etc., are not the response to events, rather than part of it. This requires genetic and transgenic approaches.

ATIS AND NOD FACTORS DO NOT SHARE THE SAME MECHANISM OF NODULE INDUCTION

Nod factor signaling, with regard to early nodulin genes, especially *enod40* expression, plant hormone perception and perturbation and cell division, may operate in different routes or pathways as simplified in Figure 5.2. Among all early nodulin genes identified so far *enod40* has drawn unparalleled high attention because of the demonstration of its possible role in plant hormone perturbation and cell division (Crespi *et al.*, 1997; van de Sande *et al.*, 1996). Study of *enod40* expression and regulation could provide clues about which route is most likely to be true. As indicated, early nodulin gene expression could be the effect or consequence of hormone perturbation or/and cell division. Recent

work by Fang and Hirsch (1997) showed that in case of *enod40*, route (1) and (3) are likely to occur, providing *enod40* expression is pertinent directly to cell cycle regulation. They found that both Nod factor and auxin transport inhibitor NPA induced nodule-like structures (referred as empty nodules and pseudonodules respectively, evidence of neo-organogenesis), and expression of some early nodulins (Hirsch *et al.*, 1989). The nodulation-specific alfalfa *enod40* gene *Msenod40-1*, however, was induced only by Nod factor but not NPA (Fang and Hirsch, 1997). It is, however, also possible that early nodulin gene expression and disturbance are events independent of cell divisions.

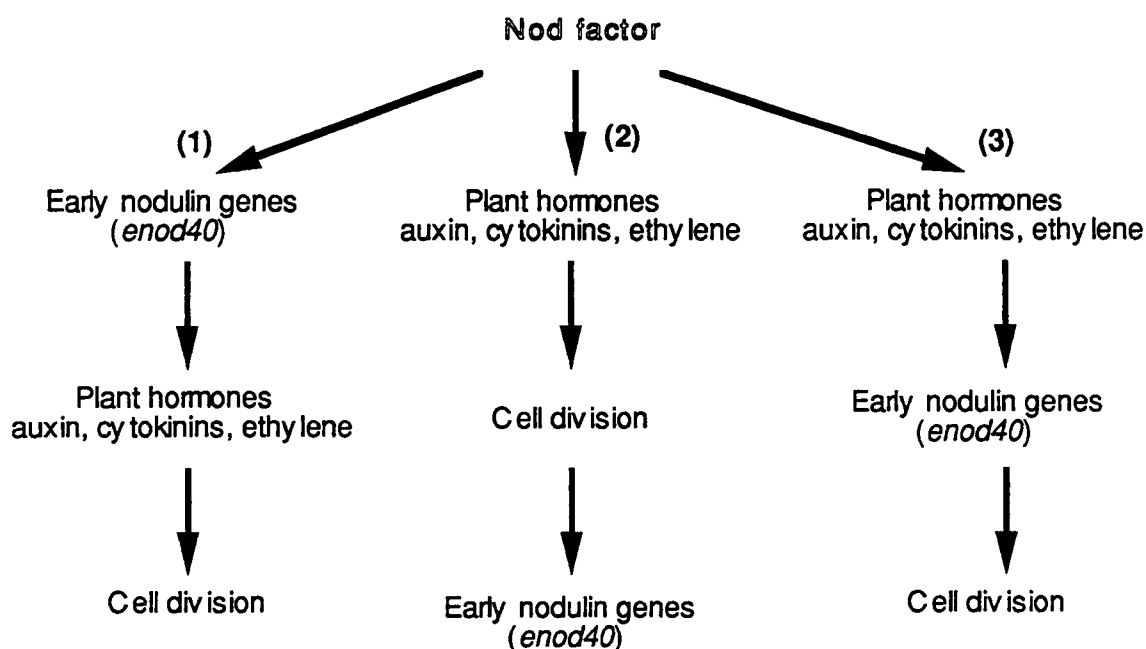


Figure 5.2. A simplified diagram of alternative Nod factor signaling in the nodule initiation (cell division). Vast possibilities exist. Plant hormones and *enod40* (and perhaps, other early nodulin genes) are assumed to be involved in the signaling pathway because modulation of hormone and *enod40* activity could trigger certain cells or tissue entry into cell cycle. It is also likely that changes in auxin and cytokinin concentrations or ratio are the consequence of *enod40* expression, or *vice versa*. Either changes in the intracellular hormone status and *enod40* expression, or both could be a result of cell division. Besides, plant hormones and *enod40* could be involved in different pathways indirectly regulating nodule formation.

Assuming *enod40* is involved in cell cycle, Yang and Hirsch's result places *enod40* gene upstream of hormone auxin perception and subsequent cell division. Because cytokinin alone can induce expression of *enod40* (Hirsch and Fang, 1994), as well as

enod2 (Dehio and de Bruijn, 1994; Silver *et al.*, 1996) in a time scale similar to Nod factor treatment, route (3) is probably the one operating *in vivo*. However, the finding does not exclude route (2) when other early nodulin genes are considered. Two implications can be drawn here. First, induction of empty nodules by Nod factors and pseudonodules by ATIs does not share common mechanism and signaling pathway. This conclusion was supported by anatomical study of pseudonodules. The pseudonodules induced by NPA on alfalfa roots were no longer cylindrical but spherical in shape. Persistent meristem was not observed in these structures. Instead of peripheral vascular system in normal nodules, the pseudonodules possess a central vascular bundle (Hirsch *et al.*, 1989). Second, if induction of *enod40* is mediated via cytokinins, mitogenic signal transduction cannot be achieved by diffusion of Nod factor or Nod factor stimulated secondary messages (Geurts and Franssen, 1996). Elicitation of nodule-like structures might reflect a general perturbation of intracellular hormone equilibrium. NPA which induce pseudonodules on alfalfa has been shown to have no effect on *L. japonicus* (Kawaguchi *et al.*, 1996), instead, nodule-like outgrowth was stimulated by exogenous applied gibberellin GA₃. Because nodule-specific *Msenod40-1* was not expressed in pseudonodules induced by NPA, the contribution of *enod40*-modulated auxin perturbation in development of functional nodules remains to be obscure.

rol* genes of *A. rhizogenes

A. rhizogenes oncogenes *rolA*, *B*, *C* and *D* (*rol* stands for root locus) are primarily responsible for the hairy root symptom (White *et al.*, 1985; Capone *et al.*, 1989). Besides, in transgenic plants or organs produced after *A. rhizogenes*-mediated transformation, unusual phenotypes were observed, such as meristematic nodules in soybean, delay of nodulation in transgenic soybean roots and spontaneous regeneration of *Lotus* plants from hairy root culture. All these have been targeted to *rol* genes. The speculation was based on abnormalities observed in other *rol* gene transformed plants (Schmülling *et al.*, 1988, 1989; Schmülling *et al.*, 1993; Dehio and Schell, 1993; Kuriota *et al.*, 1992; Nilsson *et al.*, 1996; Maurel *et al.*, 1991a; 1991b). The *rolA*, *B* and *C* genes can cause abnormal growth of plants transformed with these genes individually or in combinations have been well documented (Dehio and Schell, 1993; Maurel *et al.*, 1991c; Schmülling *et al.*, 1989). Alterations are likely the consequence of perturbed hormone balance resulted from high level expression of ROL proteins because transgenic plants carrying *rolB* or *rolC* gene were

more sensitive to plant hormones, particularly, auxin (Maurel *et al.*, 1991a; 1991b). Direct evidence to substantiate the claim that *rol* genes and their interactions with plant genes caused the altered phenotype in soybean roots (Bond *et al.*, 1998; also this thesis) and AR12-induced *L. japonicus* hairy root lines (Chian *et al.*, 1998) are definitely needed.

The induction of meristematic nodules was apparently bacterium strain-specific, but not soybean cultivar-specific. Such abnormal nodules have been observed in roots of 'Peking', 'Bragg' and its nodulation mutants *nts382* as well as cultivar 'Williams' (Dr. Peter Straube, Washington University, St. Louis, MO., personal communication), all transformed with K599 or K599 GUS-I. Because only small proportion (about 10 %) of chimeric soybean plants produced abnormal nodules on transgenic roots, it was thought that the alteration was due to the positional effect or/and copy numbers of T-DNA integration. The positional effect assumption also points to the spontaneous transgenic *L. japonicus* plants produced only from *A. rhizogenes* strain AR12-induced hairy root lines. Since the only difference between AR12 and its sibling strains AR10, AR14 and parent strain 15834 is the reporter gene integrated into T-DNA of the Ri plasmid. Insertion of reporter gene in different sites could bring about to a different expression patterns and thus plant cell responses. It is also possible that other bacterial genes residing in the T-DNA contributed to these abnormal phenotypes. In *A. tumefaciens*, a T-DNA located gene *6b* was found to interfere with bacterial oncogenes *itp* and *iaa* (for cytokinin and auxin biosynthesis, respectively) during tumor formation (Tinland *et al.*, 1989).

Such strain-specificity was also apparent in spontaneous regenerants of *L. japonicus* 'Gifu' generated from liquid root culture of *A. rhizogenes* AR12 induced hairy roots (Chian *et al.*, 1998). Regenerants were, however, not obtained from hairy roots induced by sibling strains of AR12 although the only known difference between AR12 and AR10, AR14 is the reporter genes inserted in the Ri plasmid (Table 3.2). It was speculated again that the positional effect of the inserted reporter gene might interfere with individual or integrative functions of *rol* genes.

Closing marks

The analysis of molecular mechanisms of the plant partners in legume-*Rhizobium* symbiosis research has long lagged behind the bacterial genetics. Most late nodulin genes

showed that they are involved in nitrogen-fixing processes. In contrast, the building of an environment for bacteroids accommodation and functioning is poorly understood. No clear function has been assigned to any early nodulins. Nevertheless, significant progress has been made during last decade, partially benefited from using model systems in other research areas such as *Arabidopsis*, and yeast.

Many early nodulation-specific genes have been identified. Failure to demonstrate the functions of *enod* genes probably stems partially from the redundancy of genes and partially the methods that genes were isolated. Genes isolated from specifically expressed mRNAs do not reveal information about gene context. Position effect could have great influence on expression patterns. Using insertional mutagenesis techniques such as T-DNA tagging, gene isolation can be directly linked to the phenotype the gene controls. The new transformation regeneration protocols for model legume *L. japonicus* developed by Stiller *et al.* (1997) showed high promise in isolating genes important in symbiotic nodulation. Several nodule-/root-development tagged transgenic lines are currently under preliminary characterization in our laboratory. Hopefully, nodule-specific genes will be identified with this method.

The involvement of plant hormones in nodulation has been long speculated (Libbenga and Bogers, 1974; Thimann, 1936). Now it comes back into the sight, as shown in the alteration of nodulation phenotypes by hormone antagonists (ATIs) and interactions of hormone(s) with regulatory genes (*enod40*). Knowledge in different fields of developmental biology has been integrated and applied to studies of nodule development, such as cell cycle regulation, signal transduction pathways, positional effect and apoptosis/senescence. It is anticipated that within 10 years a clearer picture of molecular aspects of nodule development will be available. The convergence of interactions of both plant and bacterial (*rol*) genes with plant hormones will help to elucidate the mechanism of hormone action in organogenesis and cell differentiation, of which nodulation consists of a unique part.

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Appendix 1.

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1  AATTCTATTA TGGATATAAA AGTAATTTGA TAATAATAAA TAATAAATAT
51  TGAAATCCCT CCCCTCACCT CCCCTCCTAT GAACCAAACA TAATAAATAT
101 TAAAATCCCT CCCCTCCCAT CCCCTTCCCT CCCCTCCCCT CCATTAAAAAT
151 CTCTCCCTC CCCTCCCTC CATTGAACCA AACACACCGT TAAAGTCCTT
201 GAAATGCCCTC TTCGAGATCA TAGTCTATAA TAAACCAACT ATGCTATTTT
251 GGGTTGGTTC TACTTTGGAC ATTTTACGG TCCACGATT AAGATATATC
301 TTTAGGACAT GAGTTGCTTA TAGATGAACT AACAAATTTGC TCATCAATAC
351 AACACTCACA TCAATTGTAG GAGTAGATTG CACAATTTTT TGATGAGAAG
401 TAATTGTTCT TAATAATTAA GCTAATATGT GCTTCTAAT ATATTCATAT
451 GTATCAAAA CAGGTTAAAT AGTACAAGAT TTGTATTATA TAATTATGGT
501 AGCTAGGTTT gTTAAAAATT TCCCATTTAA ACCAGAGTGA AAAATAGTAA
551 ATGGGGGAAT TAGGTAGGTA ATTAGGTTAA GGTAAATAT GTCATATAAA
601 CTAACATAATC CTTTATTTGT TTGTTTATTT CAATGGACAC TGTTCTGTAT
651 GTTATCTCTC ATTACTTTCT AGAATTGCAT CCTATGATAG CAAAAGAATG
701 CATTTGAATA ATGAATAGGT GATGCCATTT CTGAGTTCTC AATCATCAAC

751 TATTTAAAGC AATGATCAGA AACTAAGTTC TCCACTAGCT +1TCCCAGAGAG
801 CCATTTGGGG GAAAACCGAA CGGACAAAGT TATCCAAGGT GAATCTGTTA
851 GACATGAGAT TTTGTTGGCA AAAATCCATC CATGGCTCTT GAAGAAGCAT
901 GGAGAGGAAT GTGTGAGCGG GTCAGGGTTT TTCACCCCTC ACACACAaTC
951 CTATGCATCT TACAGTTTGT TTAGGCTTAG CCATTGGCTT CTCTAGTcTT
1001 CAAAACTCGT TAtGTTGCGG TCGCACTTGC AGTTGTGGAT CGCAATTTGA
1051 AACCCGAGTT AGTAATCACC GGGCATACCG AGAAGGAGT TTTATGAGTA
1101 GAAGAAGTTG AGAGAGAAGC TTTGGCTACA GCCTGGCGAA CCGGCAAGTC
1151 ACAGAAAGGC AATGGACCCC ATTAGGGGTT CTTATGGCTA TGTATGAATG
1201 ACTCTTTTGT AGTTCTTCTT CAAGTAGAAT GTAATAAACA AAAATGTTCT
1251 TCTTCCTTTG AGGTGGTGTT CATTCAATATC ACCCAATTTG CAGCTGACTA
1301 GACTCCAGTT TGCTCTTCAG TTTCTGCAGA TGAGTAGGTA GGTAAATTATG
1351 ATCACCTTGT CTCTTCTTTT TCTGTGTCTG TTTTCCTTTT CTCATGTGTG
1401 TATGGTTGTT GTCTTGTGGC CTTATATGCA ATAATAGTAT TTGAGTGATG
1451 TTTTTGTTCC TTCAAGTGTA GGATTGTGTT TTGTAGAAGT TGATCGAGTC
1501 GACTCCCTTT AGTGAGGGTT AATTGAGCTC GCGGCCGCGA GCTCTAGAGA
1551 TCCACTCGTT A

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Total length:	1561
TATA box:	751
CAAT box:	740
Alternative CAAT box:	725
Transcription startpoint:	792
Translation start codon:	854
ORF:	854 - 889
cds:	792 - >1561

Appendix 1. The full length of *Ljenod40* gene. The putative transcription startpoint (labeled +1 on top of the base), TATA and CAAT boxes (underlined), and ORF (bold in initial and stop codons) for a short peptide of 12 amino acids are indicated.

Appendix 2.

Publications from this study

Stiller, J., Martirani, L., Tuppale, S., Chian, R., Chiurazzi, M. and Gresshoff, P.M. 1997. New transformation regeneration protocols and perspectives of insertional mutagenesis in the model legume *Lotus japonicus*. *Journal of Experimental Botany* 48 (7): 1357-1365.

Bond, J. E., Joshi, P., Chian, R., McDonnell, R. and Gresshoff, P.M. 1998. Programmed cessation of cell division is reversed in transgenic soybean nodules. (submitted)

Chian, R., Stiller, J. and Gresshoff, P.M. 1998. Spontaneous regeneration of plants without somatic embryogenesis from *Lotus japonicus* hairy roots inoculated with *Agrobacterium rhizogenes* AR12. (submitted)

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VITA

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